TRANSITION STATES IN MULTICYTOKINE RESPONSIVENESS DURING THE PROGRESSION OF HUMAN CUTANEOUS MALIGNANT MELANOMA

by

Chao Lu

A Thesis submitted in conformity with the requirement for the Degree of Doctor of Philosophy, Institute of Medical Science in the University of Toronto, Toronto Ontario, Canada

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This thesis is dedicated to people who work hard to fight against cancer in both basic medical research and clinical treatment, and to cancer patients and their family members who understand what the cancer means to them.
Transition states in multicytokine responsiveness during the progression of human cutaneous malignant melanoma.

Chao Lu, for the Degree of Doctor of Philosophy, 1997,

Institute of Medical Science of University of Toronto, Ontario Canada.

Human cutaneous melanoma sometimes may progress through well-defined clinical stages, from normal melanocytes, *via* benign nevi, early-stage radial growth phase (RGP) and "thin" radial growth phase (VGP) melanoma (metastatically-incompetent), towards "thick" VGP (metastatically-competent) and metastatic melanoma. Melanocytic cells from different clinical stages have been successfully cultured *in vitro* for studies on tumor progression. It has been shown that normal dermal fibroblastic cells inhibit the proliferation of early-stage RGP and thin VGP melanoma derived cell lines whereas this inhibitory effect is lost on advanced-stage metastatically competent melanomas. In this thesis, evidence has been obtained showing that one molecule that is responsible for such differential growth regulatory activity is interleukin (IL) 6, a pleiotropic cytokine. Results also indicate that the loss of responsiveness to IL-6-induced growth inhibition in advanced-stage melanomas is not due to a lack of receptors, based on northern blotting and ligand binding Scatchard analysis. Studies on IL-6 gene expression by northern analysis and on IL-6 protein production by B9 cell bioassay and ELISA of culture medium of melanoma cells revealed that about half of IL-6 resistant cell lines, and none of the sensitive cell lines, produce biologically active IL-6. Attempts to down-regulate IL-6 production by use of antisense strategy with cDNA stable transfection were undertaken. The results indicate that the endogenous IL-6 can function as
a growth stimulatory factor in advanced-stage melanoma cells that have spontaneously acquired endogenously IL-6 production. A similar resistance was also found in advanced-stage melanomas with respect to the growth inhibition induced by several other cytokines, such as IL-1α, IL-1β, tumor necrosis factor α, and oncostatin M. This phenomenon was called "multicytokine resistance". In summary, it is shown that there is a transitional change of IL-6-regulated cellular and tumor growth in human melanoma cells during disease progression, from a cell growth inhibition, to growth resistance, and finally a tumor growth stimulation. Given the possible presence of abundant levels of host or tumor-derived IL-6 in melanomas in vivo, these effects may provide rare malignant melanoma cells with a growth advantage in the primary site allowing them to become the eventual dominant population.
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INTRODUCTION

1. DISEASE PROGRESSION OF HUMAN CANCERS

i): Multistep development of human cancers;

Cancer is one of the leading causes of death in human beings. Human cancer is, as defined by Clark (81), "a population of abnormal cells showing temporally unrestricted growth preference (continually increasing numbers of cells in the population) over their normal counterparts. Such abnormal cells invade surround tissues, traverse at least one basement membrane zone, grow in the mesenchyme at the primary site, and may metastasize to distant sites. It is the totality of properties, not any one property, that determines whether or not a given lesion should be designated as a cancer". There are over a hundred different kinds of cancer, at least one originating from nearly every cell type in the mammalian organism. In the case of the homeopathic system, normal cells have the capability to traverse the endothelial basement membrane zone, and this property is not necessarily acquired as a new phenotype during malignant disease progression. Instead, "unrestricted growth preference" is probably the most important phenotype of malignant cells, the result of an accumulation of multiple changes at the cellular and molecular levels, leading to disease progression and eventual competence for distant metastasis (110,180).

Evidence for the multistep nature of cancer development and progression has been obtained from a variety of "models" including human colorectal cancer, melanocytic neoplasia, and as well as in various experimental rodent models of hepatic and skin neoplasia,
to cite some of the better known examples (81). The arguments for the transition of benign
to malignant tumors are based on the following observations (81,312,401): (i) benign tumors
are commonly seen in patients with their adjacent malignant counterpart while some malignant
lesions are surrounded by benign tumors; (ii) the peak age of benign lesions usually precedes
that for the corresponding malignant cancers; (iii) animal experiments under controlled
conditions have provided strong evidence which show the sequential change from normal
cells, to dysplasia, to benign tumors and finally to malignant tumors. Such malignant
transformations are frequently accompanied by defined genetic alterations.

Colorectal cancer is among the most common of cancers in North America. Currently
nearly 15% of all human cancers diagnosed originate in the colon or rectum (138). The
incidence during the past few decades has been rising steadily, (partly because of better
diagnostic procedures) while the mortality rates remain flat with a significant increase in 5-
year survival rates (138). It appears that some colorectal cancer develops sequentially from
a benign precursor lesion, the colorectal adenoma or polyp, to malignant colorectal
carcinoma, i.e. the adenoma-adenocarcinoma sequence, and that this is accompanied by a
variety of molecular genetic abnormalities. These include mutational inactivation of recessive
tumor-suppressor genes and mutational activation of dominantly active oncogenes
(81,110,154). Normal epithelium is of polyclonal composition, among which a single pocket
of epithelial stem cells can give rise to a monoclonal adenoma. Depending on the size of these
lesions, adenomas have been classified as early, intermediate and late stage. Early-stage
adenomas have been defined as those that are 1.0 cm or less in size. Intermediate-stage
adenomas are those greater than 1.0 cm in size and do not contain foci of carcinoma, whereas
late-stage adenomas are greater than 1.0 cm in size and contain foci of carcinoma (110,312). Evidence has been accumulated to support the hypothesis of a stepwise transition from adenomatous polyps to carcinoma. Certainly, not all polyps undergo malignant transformation (in fact the vast majority do not) but it seems that the more polyps that are allowed to grow to larger size, the greater the likelihood of cancer developing from one or more of them (401). Small polyps and adenomas are considered to be a biologic indicator, or early preclinical phenotypic characteristic, that relates to future risk for developing colorectal carcinomas (312).

In addition to human colorectal tumors, a similar step-wise phenotypic development of cancer has been observed in human cutaneous keratinocytic neoplasia and cutaneous melanocytic neoplasia (81). The malignant proliferation of keratinocytes of the epidermis, i.e. the squamous or basal cell carcinomas, are the most common cancers in much of the world. The main risk factors are related to cumulative sun exposure and the degree of pigmentation (367). Cutaneous squamous cell carcinomas may develop from pre-existing cutaneous dysplasia. The common initial lesion is a benign keratosis, i.e. a circumscribed area of thickening of the epidermis which is not papillary in form (81,367). Aberrant differentiation of the initial lesion and dysplasia are characterized by persistence of the initial lesion, mitotic activity above the basal layer and atypical keratinocytic hyperplasia (81). These can sometimes progress into squamous cell carcinoma in situ, the prototype of "intermediate" lesions and such lesions may eventually develop to invasive squamous cell carcinomas (81,367). Tumor cells at the intermediate stage are generally confined above the basement membrane zone and are not associated with any apparent competence for metastasis (81).
Squamous cell carcinomas may invade deeply into the reticular dermis, but rarely give rise to metastases (81).

Human cutaneous melanoma appears to be another good example of the stepwise development of human cancer, in this case from normal melanocytes, through atypical benign nevi, and then on to malignant melanoma. This will be discussed below in more detail, as human cutaneous melanoma was the "model" I chose for my studies, the results of which are described in this thesis.

ii): Genetic alterations in human colorectal tumors

Knowledge of some genetic alternations may help us understand the molecular basis of the disease progression of human cancers. The best characterized example is human colorectal cancer. Meanwhile, some of the genetic abnormalities found initially in colorectal cancers have been observed in many types of other human cancers. It is therefore thought necessary to briefly discuss these genetic changes during tumor progression of colorectal cancer, which may be helpful in understanding the significance of a similar changes in human melanomas.

The most prominent cellular phenotype of cancer cells is their unrestricted progressive growth, especially in ectopic tissue sites. This acquired trait is due to various underlying genetic alterations, which provide successive tumor cell clones with a growth advantage, allowing them to overgrow normal cells and precancerous lesions. Activation of oncogenes and inactivation of tumor suppressor genes are among the major genetic alterations found in cancer cells (41,42,180,241,268,355,453).
Oncogene products range from nuclear transcription factors, plasma membrane receptor kinase proteins to secretable growth factors, and constitutive activation or overexpression of these oncogene (or proto-oncogene) products can initiate unrestrained DNA synthesis, abnormally regulate the transcription of gene expression and generate constitutive signals of cellular proliferation by catalyzing phosphorylation of cytoplasmic substrate (3,14,41,180,241). Although this has been considered to be one of the main ways such genetic alterations drive disease progression, there are also additional possibilities. For example, ras gene mutations can upregulate the production of the paracrine angiogenesis growth factor known as VEGF/VPF (139,337).

The normal product of most tumor suppressor genes is thought to function mainly as a negative growth regulator to maintain the homeostasis of tissues through the interaction with positive regulators. Loss of the function of tumor suppressor gene products, such as p53, or pRB has been well documented in many types of human cancer (268,355,453). In particular, the strong relationship of accumulated genetic alterations, including inactivated tumor suppressor genes, and mutant oncogenes with tumor progression has been most thoroughly documented in human colorectal tumors (110).

Extensive molecular and genetic studies have revealed some of the genetic basis for multistage development of human colorectal adenomas and their transition to carcinomas. These genetic alterations include (but are not limited to) DNA hypomethylation, mutation and/or deletion of genes such as \textit{apc}, \textit{dcc}, \textit{ras}, and \textit{p53} (110).

The \textit{apc} gene (for \textit{Adenomatous Polyposis Coli}) located in the long arm of human chromosome 5 (5q21) was identified from cytogenetic studies of a patient with nonfamilial
adenomatous polyposis. The predicted product of the \textit{apc} gene is expected to be a cytoplasmic protein that has a short segment of homology with a yeast gene that appears to regulate the \textit{ras} oncogene and another short segment of homology with the critical G proteins-binding region of the m3 muscarinic acetylcholine receptor (154). Alteration of the \textit{apc} gene (by mutation and/or small deletions) has been identified in most if not all patients with familial adenomatous polyposis (FAP) and in some sporadic colorectal carcinomas (110,154,332,387,400). The mutant form of \textit{apc} product is able to interact with wild type \textit{apc} protein, and this in turn may inactivate the wild type \textit{apc} protein through a dominant negative effect (387,400). It is believed that mutation of the \textit{apc} gene contributes to the early development of the colorectal neoplasms, probably at the epithelial hyperproliferation (dysplasia) stage (110,154) by virtue of the fact that mutations in this gene were found in the earliest tumors that could be analyzed; moreover, the frequency of such mutations remain constant as tumors progress from benign (63%) to malignant stages (60%) (332).

Another mutation that is commonly detected in the early-adenoma involves \textit{ras} genes (H-\textit{ras}, K-\textit{ras} and N-\textit{ras}) (110,154). The \textit{ras} gene family encodes highly similar 21 kDa membrane-bound GTP-binding proteins that are believed to be involved in several signal transduction pathways, such as \textit{raf}, \textit{rho}, \textit{rac}, and RHAMM to induce cell proliferation, reduce programmed cell death, or apoptosis, and upregulate genes encoding potent angiogenesis growth factors, such as VEGF/VPF, resulting in transformation of cells and even metastasis (41,71,152,334,337,338,486). Mutated \textit{K-ras} genes are found in 90% of adenocarcinomas of the pancreas, 50% of colorectal adenomas and adenocarcinomas and thyroid tumors, and 30% of myeloid leukemias (49). They have been detected in both
carcinoma tissues and late stage (larger, villous type) adenomas, and also in some of the smaller, tubular type of adenomas, indicating that such mutations usually occur before the conversion of malignant carcinoma, and thus are partly responsible for further progression of the disease (49,110,154).

Deletion on the long arm of chromosome 18 is also frequent in adenocarcinomas of the large bowel, but less so in the adenomas from which they presumably arose. The relevant gene in this region was termed \textit{dcc} (for Deleted in Colorectal Carcinoma). It is predicted that the gene product is 190 kDa with some homology to neural cell adhesion molecules and consensus transmembrane domains (110,154). Evidence for allelic loss of the \textit{dcc} gene has been found in 70% of colorectal carcinomas and in almost 50% of late adenomas (110). Furthermore, it was found that the \textit{dcc} gene is expressed in normal cells, such as colonic mucosa, or normal prostatic cells, but its expression was reduced or absent in the majority of the colorectal carcinomas and prostatic carcinomas examined (110,123).

The most common region of allelic loss in human colorectal carcinomas is in chromosome 17p (110,173). This region contains the \textit{p53} gene (17p13.1), now known to be one of the tumor suppressor genes, whose product is a 53 kDa nuclear phosphoprotein involved in the control of cell proliferation, differentiation, and cell survival (110,173,284,355,453). Experimental introduction of the wild type \textit{p53} gene has a negative effect on tumor cell growth, and can suppress cell transformation brought about by other oncogenes, such as \textit{ras} (284). There is evidence to show that 17p allelic losses are associated with the progression of individual tumors from adenoma to carcinomas. Allelic loss and deletion of the wild type \textit{p53} gene is found to be frequently coupled with point mutations of
the other $p53$ allele (110,173). Mutations in the $p53$ gene affect the conformation of the p53 protein including alterations of the structure of domains located far from the mutation sites. These changes in turn affect the wild-type p53 protein which forms complexes with mutant p53 proteins. Such a conformational change of p53 protein in the complex results in a loss of binding sites, causing the inactivation of wild-type p53 protein in a "dominant negative" fashion (444,483). Studies have shown that alterations of the $p53$ gene are detected less commonly in benign tumors, and much more frequently in late stages of disease progression in many types of cancers (110,236,266,268,413,444,453). This being the case, it may be more logical to designate $p53$ as a "malignancy/metastasis suppressor" or "progression suppressor" gene, as opposed to a "tumor suppressor" gene - at least in those instances where it occurs as a very late event in the evolution of malignant tumor progression.

In addition to the aforementioned genetic alterations, there are a number of other known (and, presumably unknown) genetic abnormalities which can occur during the development of human colorectal carcinomas (1,110,154,258,323,370). Fearon and Vogelstein originally pointed out that it is the accumulation, not the order of the genetic changes that is important and which result in the successive waves of clonal expansions leading to primary carcinoma and subsequently metastasis formation (110).

iii): Human melanoma as a model for the study of the sequential stages of disease progression in human cancer.

Melanomas, tumors of the melanocytic lineage, were described as early as the 5th century B.C.. They are among the most malignant of all cancers (84,220). The vast majority of melanomas occur in the skin, i.e. are cutaneous. The incidence of human cutaneous
melanoma has increased significantly during the past few decades in both Australia, Europe, as well as in north America, especially in Caucasians (117,118,133). The incidence of melanoma rises with age from the second decade (84,133). Risk factors for cutaneous malignant melanoma include certain (acute) types of exposure to ultraviolet radiation especially in early age, and the presence of melanocytic nevi, both clinically benign and atypical (dysplastic). The strong correlation of melanoma with melanocytic nevi in some patients has led to the notion that atypical melanocytic nevi may be the preneoplastic precursor lesions of cutaneous melanoma (81,133).

The melanocytic nevus appears initially as a flat, tan dot. Enlargement occurs slowly at its periphery and gradually becomes elevated. Thereafter these lesions almost always slowly flatten and spontaneously disappear. A very small proportion of these precursor lesions may eventually progress to the first stages of malignant melanoma development (81,160).

During the earliest stage of cutaneous melanoma development, called the "radial growth phase" (RGP), the primary tumor undergoes centrifugal enlargement. The lesions tend to be relatively flat and their outlines may be irregular, but the overall shape is circular to oval. This RGP period may persist for up to years. The growth of tumor cells is mainly restricted within the epidermal tissue compartment. Occasionally, some of the tumor cells may break the epidermal/dermal basement membrane and invade into the papillary dermis in the absence of significant proliferation. This is called the "invasive" RGP or "in situ" melanoma. It is generally believed that human RGP melanomas or invasive RGP melanomas do not have the competence for metastasis. Hence, surgery performed on primary RGP melanoma is almost always curative (81,84,144,160).
The next phase of development is the vertical growth phase (VGP), which appears as a focal nodule within an otherwise flat lesion; it presumably represents a new and distinct subclone of tumor cells. The dome-shaped VGP nodule grows more rapidly than its RGP precursor lesion, and it is during this invasive phase of growth that the tumor penetrates the underlying connective tissue, gaining access to blood and lymph vessels, thus increasing the probability of distant metastases. An important feature of VGP melanoma is that the tumor cells have the capability to readily proliferate in the dermis, which significantly differs from the invasive radial growth phase melanoma. Acquisition of this proliferative capability in the dermis is considered to be the key step of disease progression towards metastasis (81,144).

Clark's levels of invasion of human cutaneous melanoma is a widely accepted system of measurement of depth of invasion used for making prognosis. Level I melanoma refers to those primary tumors with restricted growth in the epidermis (above the basement membrane), called the RGP, whereas level II tumors tend to invade the basement membrane into the loose papillary dermis. RGP and most if not all the level II VGP (sometimes called "thin" VGP) rarely give rise to metastases, i.e. appear to be metastatically incompetent, and patients with such tumors usually have excellent 10-year survival rates (over 95%) (27,84,144,220). However, primary melanoma of level III or greater (called "thick" VGP) have a marked tendency for metastasis through blood vascular and/or lymphatic pathways (27,81). For patients presenting with primary melanomas of more than 3.5 mm in thickness, the long-term survival rate is only 33 to 48 % (81,84,133). Besides the correlation with clinic survival rate, the vertical thickness of the primary tumor can also be used for clinic prognosis. Breslow's thickness classification of 0.76 mm has been considered to be the critical dermal
level; patients with cutaneous melanoma less than 0.76 mm would have much better prognosis whereas those with thicker than 0.76 primary melanoma have worse prognosis. Therefore, the most accurate and predictive factor for the recurrence and prognosis of melanoma is the vertical size of the primary lesion followed by the depth of tissue invasion of the original lesion of the tumor; the long term survival rates of patients decreases with increasing thickness in an almost linear fashion (27,81,133).

In stark contrast to human colorectal carcinomas, the underlying genetic alterations that are associated with disease progression in human melanomas are unknown. Chromosome studies of human melanocytic tumors have demonstrated non-random karyotypic abnormalities of chromosomes 1, 6, 7, 9, and 10 (160,228,318). Introduction of a normal copy of chromosome 6 could suppress the tumorigenicity in some human melanoma cell lines (346). These visible genetic alterations may eventually provide clues to the location of oncogenes and tumor suppressor genes involved in the tumor development from benign nevi to metastatic melanoma. Nevertheless, there have been reports documenting constitutive expression of some proto-oncogenes in malignant melanocytic cells, such as c-myc, c-fos, c-neu, Ha-ras, c-kit, c-ski and p53 (76,120,182,236,301,302,325,399,482). The proto-oncogene bcl-2, whose product functions to inhibit programmed cell death, or apoptosis, is expressed not only in normal melanocytes but also in both benign and malignant lesions (328,422). Expression of c-myb was also found in melanoma cell lines, albeit at low levels, and administration of antisense oligonucleotides was found to suppress myb gene expression and reduce the growth of transplanted melanoma cells \emph{in vivo} (166).

The roles of mutated form of ras and p53 in human melanoma development and
progression are not yet clear. In terms of ras gene mutations, a study by Shukla et al. indicated that mutations of K-ras occur in all benign and malignant melanocytic lesions, as early as in benign nevi (381), whereas Wagner et al. found ras mutation in only 2 out of 50 primary melanomas from Clark's level I to level IV (447). There are experimental data which have shown that transfection of ras or infection of viral Ha-ras of normal melanocytes can induce the malignant phenotype (8,102). Moreover, other studies have indicated that ras mutations in human melanoma are associated with the depth of tumor invasion (24).

Mutations in the p53 gene, as analyzed by DNA sequencing, have indicated that they are rare in melanomas, or in melanoma cell lines (101,256,445,455). However, immunohistochemical staining using antibodies specific for mutated p53 have revealed a very high degree of staining in human melanomas (236,399,455), the extent of which appears to correlate with disease progression (236,399). Thus, p53 may be functionally abnormal or inactivated in malignant melanoma; if so, the abnormality occurs in the absence of simple point mutations that are detected in so many other types of human cancer. A report on γ-irradiated growth inhibition showed that wild type p53 in melanoma may have an abnormality in its ability to induce downstream effector genes such as GADD45, CIP1/WAF1 (21). CIP1/WAF1 is also known as p21, a cyclin-dependent kinase inhibitor, or SDI1, CAP20, or mda6 (135,194). Interestingly, it was discovered independently by Jiang et al. that the expression of p21mda6 correlates inversely with melanoma progression, i.e. it is high in normal melanocytes and in the benign nevus, whereas it is low in metastatic melanomas (194). It is known that the p21CIP1/WAF1/mda6 gene expression is highly regulated by both the p53 dependent mechanisms and some p53-independent pathways (135). Another cyclin-dependent kinase inhibitor p16
has also been found to play certain role in the development of human melanomas, especially the familial melanomas. The p16 gene that is localized on chromosome 9p21 is found to be deleted or mutated in familial melanomas (47,140,183). However, the contribution of such abnormalities to melanoma progression still remains unclear since p16 deletion was found in both normal melanocyte isolates and in benign nevi (449).

iv): Cancer metastasis:

Tumor metastasis refers to the process by which cancer cells disseminate and form new foci of tumor growth at noncontiguous sites. Clinical observations have indicated that metastases from certain types of tumors tend to occur preferentially in specific target organs. Generally speaking, lungs, liver, lymph nodes, bone and brain are the most common sites of metastatic spread. In 1889, Paget, having examined the autopsy records of 735 patients who died of breast cancer, proposed the "seed and soil" hypothesis to explain the non-random dissemination patterns of certain human malignancies, in which he postulated that differential and selective tumor-cell/host-organ interactions exist. Such interactions result in the growth of metastatic tumor cells (the "seed") in proper secondary sites (the "soil") and thereby give rise to metastatic tumors. In recent years, some researchers have challenged the "seed and soil" hypothesis on the basis of the fact that organs with more blood flow would have a greater chance to become a secondary site for metastatic tumor growth. Clinically, both situations seem to occur in that some metastatic tumors can colonize a wide variety of tissues (the first site encountered is usually the most common site of metastasis) whereas some tumors display an organ-specific pattern of metastasis independent of blood flow.
characteristics (211,244,456,485).

The process of solid tumor metastasis includes multiple steps of tumor-host interactions (190,211,244). Cancer cells first have to detach from the primary tumor mass, and gain entry into either the lymphatic or vascular (haematogenous) systems in order to be transported by the circulation to distant organ sites. During this transportation process, cancer cells have to be able to survive mechanical stresses, non-specific immune effector cells and specific immune defense. At secondary sites, cancer cells need to exit the circulation by penetrating the endothelial cell lining and subendothelial basement membrane (extravasation), and then settle in new tissue sites. Whether a particular cancer cell can proliferate to generate a secondary tumor depends on whether the organ is a favourable site for the cancer cell, i.e. the seed and soil relationship supposedly determine the organ specificity of tumor metastasis.

In reality, the process of metastasis is very inefficient and the vast majority of the cancer cells entering the multistep metastatic process do not form metastases, i.e. the attrition rate is extremely high (456). Therefore, the potential ability of cancer cells (the seed) to metastasize is necessary but not sufficient for the accomplishment of the entire process of metastasis. Since only a minority of cancer cells metastasize, a question which arises is whether the remainder of the population of cancer cells have the potential to metastasize. On the basis of a series of experiments in which mouse B16 melanoma cells were subjected to a series of sequential in vivo and in vitro selections, it was proposed by Fidler that cancer cells in solid tumors were heterogenous with respect to metastatic potential, and the metastases arose exclusively from subpopulations having a preexisting competence for metastasis that were present in the primary tumor. However, some clinical and experimental data do not
always support the hypothesis that only minority subpopulations in the primary tumor have the competence for metastasis (211,244). Since then a number of other models have been introduced to account for the evolution of tumors from a non-metastatic to metastatic phenotype.

The "clonal dominance" hypothesis proposed by Kerbel and his colleagues (211,214) suggested that during the early stage of tumor development, the vast majority of tumor cells are not competent for metastasis but as the disease progresses, a subpopulation of metastatic tumor cells emerge (because of underlying cellular and genetic changes). These cells can manifest a growth advantage at the primary tumor site and thus gradually overgrow the primary tumor, as well as giving rise to metastases. In such a scenario it would not be surprising to find that the properties of "advanced" primary tumors and distant metastases would be frequently found to be identical or very similar; the same would not hold true when comparing the properties of early stage primary tumors with distant metastases.

Metastatically competent tumor cells are characterized in part by their ability to adhere to the microvascular endothelial cells of the target organ, respond to chemotactic signals emanating from the target organ, attach to, invade, and degrade the subendothelial matrix of the target organ, evade the immune surveillance systems of the target organ, and respond to local growth signals in the target organ. During such tumor-host interactions, metastatically-competent tumor cells should presumably acquire resistance to negative growth regulatory factors and to be able to respond to mitogenic paracrine factors made by other types of adjacent normal cells. A stimulatory activity on tumor cell growth by normal or stromal cells has been detected in several malignant systems (63,69,132,326), whereas a negative growth
Regulation is found in some other systems (34, 88, 103). Some of the paracrine growth regulators produced from normal cells for cancer cells have been identified. For example, a growth factor purified from lung tissue on the basis of its ability to stimulate differentially the proliferation of lung-colonizing murine B16 melanoma cells was shown to be a transferrin-like mitogen (5, 309). Interestingly, the stimulatory activity for human prostatic carcinoma cell growth present in human bone marrow conditioned media was also found to be transferrin (69, 352). Therefore, the responsiveness of tumor cells to growth regulators produced by normal cells at different organ sites appears to be an important factor to help explain some aspects of metastasis, including organ-specific patterns of disease (308).

2. THE ROLE OF GROWTH FACTORS IN THE DEVELOPMENT OF HUMAN CANCERS

Multicellular organisms have highly coordinated mechanisms that control cellular interactions. These complex signalling networks are essential for normal embryonic development, immunity, inflammation, and haemopoiesis, and for systemic responses to wounding and infection, which is accomplished in part by soluble mediators of protein product, called growth factors, cytokines and chemokines (3, 390). The difference between cytokines and growth factors is not clear cut, with the exceptions that growth factors often tend to be constitutively expressed i.e. are not as tightly regulated as cytokines, and the major targets of such growth factors are non-haematopoietic cells. However, this is by no means a hard and fast rule and there are many more similarities among cytokines, growth factors and chemokines than there are differences (438, 461).
These growth factors and cytokines function as both positive and negative modulators of cell proliferation and differentiation, through interactions with specific membrane receptors triggering a cascade of intracellular biochemical signals (3,89). Such growth factors, to cite a few examples, include platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factors (FGF), insulin-like growth factors (IGF), and transforming growth factor (TGF) β. Most if not all growth factors were named based on their initial biological activity that was observed and assayed. Cytokines are key players in the control of cell proliferation, differentiation and survival of cells associated with the function of haematopoietic and immune systems. They include the colony-stimulating factors (CSF) and interleukins (IL). These cytokines are responsible for the intercellular communications in haemopoiesis and immunity, and some are known to also affect non-haematopoietic cells under both normal physiologic and pathologic conditions (378,438). Follow-up studies after their initial identification almost always revealed that these growth factors and cytokines are biologically active in a wide range of epithelial and mesenchymal cell types in a broad and diverse array of functions (378,438).

Cancer researchers have discovered numerous links of altered growth factor function with respect to the development of animal and human cancers (58,89,180). The B chain of PDGF is a product of the proto-oncogene c-sis, while the v-sis oncogene encodes an altered version of the B chain. Some of these growth factors have an altered form of receptors, such as erbB, fms and kit for EGF, macrophage-CSF, and steel factor, respectively (58,180). In respect to growth control, the function of growth factors as either a growth stimulator or inhibitor depends on many factors, such as target cell type, cell lineage, and physiological
status of the cell (89). Some small peptide regulatory molecules are also involved in the development of human cancers (462), but a discussion of such factors is not within the scope of this thesis.

i): Proliferative stimulation by growth factors:

The stimulation of cell proliferation by growth factors can cause cells in the resting or G0 phase to enter and proceed through the cell cycle. The mitogenic response occurs in two stages: in the first, quiescent cells must advance into the G1 phase of the cell cycle with the assistance of "competence" factors, traverse the G1 phase, and then become committed to DNA synthesis under the influence of "progression" factors. Transition through the G1 phase requires sustained growth factor stimulation over a period of several hours. Removal of growth stimulatory factors before cells pass the G1 restriction point can cause cells to revert back to the G0 phase (3,180). Growth factors in one group (e.g., PDGF and FGF) can convert the cells into a competent state, and the competent cells can then respond to other growth factors belonging to the second group (e.g., EGF and IGF-1), which stimulate the cells to progress through the cell cycle. Presumably this dual signal requirement is a form of fail-safe mechanism to prevent accidental triggering of quiescent cells into cycle by transient exposure to mitogenic growth factors (180).

Growth stimulatory factors exert their biological activities through the binding to specific receptors, which results in activation of the receptor. This in turn causes the activation of other intracellular regulatory proteins in the signalling pathway. Such signals then are conveyed to the nucleus, where the expression of specific growth control genes is
induced (or repressed), resulting in cell division.

Many of these growth factors have specific membrane receptors with an intrinsic protein tyrosine kinase activity. Growth factor receptors with protein tyrosine kinase activity, or "receptor tyrosine kinases", have a similar molecular topology. A number of these receptor tyrosine kinases are encoded by proto-oncogenes which are frequently over-expressed in cancers, such as the EGF receptor and erbB2-neu (3,41). The enzymatic activity of receptor tyrosine kinases is normally dependent on ligand binding. It appears that ligand binding to the receptor can cause a subsequent conformational alteration of the extracellular domain, and induce receptor dimerization. This stabilizes interactions between adjacent cytoplasmic domains and leads to the activation of kinase function. Such oligomerization of growth factor receptors seems to be a universal phenomenon and is found in all living cells, isolated membranes and preparations of solubilized and purified receptors (428).

There are also other cytoplasmic tyrosine kinases, such as p21GTPase-activating protein (GAP), src and src-like tyrosine kinases that are likely associated with growth factor membrane receptors (224). These proteins have quite different enzymatic properties and are structurally distinct within their catalytic regions. However, with the exception of raf, they share conserved noncatalytic domains termed src homology (SH) regions 2 and 3, which play a pivotal role in protein interactions with receptor tyrosine kinase. Proteins with SH2 domains frequently have SH3 domains (224,321). SH2 domains recognize and bind to phosphotyrosine residues within specific amino acids sequences. Several non-receptor protein kinases have been found to have SH2 and SH3 domains, which constitute part of the basis of interaction among cytoplasmic signalling proteins. The existence of different binding ability to
phosphorylated tyrosine of some receptor kinases by particular SH2-containing non-receptor kinases suggests a way to achieve the relative specificity of growth factor-induced cellular responses (64, 224).

Other functional domains, such as the PH (pleckstrin homology) domains have also been found to play a very important role in signalling transduction. Many signal transducing proteins, including PLC, Shc, IRS, contain in their molecular structure the PH domain or different subclass of PH domain such as PTB (phosphotyrosine binding) domain. The potential ligand of specific PH domains includes various inositol polyphosphates, phosphorylated membrane components and phosphorylated tyrosine, serine, threonine residues (239).

The ultimate target of many mitogen-activated signal pathways is transcriptional activation of a specific set of genes that are related to cell cycle control. By comparing molecular structure and function, it is known that there are several classes of nuclear proteins which regulate the transcription of target genes. These transcription factors include those with leucine zipper DNA binding domains (jun and fos), helix-loop-helix structures (myc), zinc fingers (erbB and gli1), homeobox (pdbx) and other specific domains (ets1) (180).

The cell regulation pathways initiated by growth factors may be subverted at several distinct levels in cancer cells. The abnormal production of a growth factor may, through action on its cognate receptor, stimulate aberrant growth. The oncogene products sis (PDGF-B chain) and int2 and hst (FGF), for example, are likely to be growth factors which operate by an autocrine mechanism. Since the corresponding growth factors, such as PDGF and FGF do not transform cells when added exogenously, the stimulation of intracellular forms of
receptor by the proto-oncogene product may have an important role in cell transformation (180).

The need for a growth factor to drive proliferation can also be bypassed in several ways, for example, by the expression of abnormal receptors which no longer require their ligand to generate a signal, or through the activation of post receptor processes within the intracellular pathways which normally transduce and modulate the receptor signal. Oncogenes also encode mutant forms of cell surface growth factor receptors, mostly the receptor tyrosine kinases. Some of the mutant receptors encoded by oncogenes have known ligands, such as EGF and M-CSF for v-erbB and v-fms, respectively (56,180,450). Trk is considered to be part of the NGF receptor (26). However, some oncogenes, like ret and ros do not have functions for known growth factors (56,180,450). Another class of oncoproteins are non-receptor protein kinases associated with the inner face of the membrane, such as the src family, or cytoplasmic and soluble forms with kinase activities, such as mos, cot, and raf (45,64,180). Constitutive activation of these oncoproteins would deliver a continuous and ligand-independent mitogenic signal to the cell cycle control machinery and stimulate cell division, a process known to occur in cancer cells (56,64,67,157,180,450). It is now thought that certain oncogene, such as mutant ras can promote cell proliferation by deregulating components of the cell cycle control machinery, such as upregulation of cyclin D1 (114).

The mediators of the last step of signalling are the nuclear proteins that regulate gene expression. Mutations have been found in the proto-oncogene proteins that have transcriptional regulatory activities, such as in jun, fos and myb (180). The expression of some of the cellular nuclear proteins, such as c-myc, c-jun and c-myb, is rapidly induced in resting
cells upon stimulation with mitogens. Constitutive expression of these nuclear protein would therefore be expected to incite continuous cell cycling (180).

Studies of the proto-oncogenes and oncogenes have revealed that their protein products may function as growth factors, growth factor receptors, cytoplasmic protein kinases and transcriptional regulators; alteration of any of these could induce an abnormal cell growth state (14,41,42,64,180,241,270,273), and contribute to the development of precancerous, cancerous, and malignant growth. Before concluding this section, however, it is worth drawing attention to a new concept of how oncogenes or proto-oncogenes can contribute to the development of solid tumors in a very powerful but indirect way, namely by regulating genes encoding paracrine growth factors which induce tumor angiogenesis, such as vascular endothelial cell growth factor (VEGF) (334).

ii): Inhibition of cell proliferation by growth factors:

In addition to stimulatory effects on cell proliferation and control of cell differentiation, growth factors can also act as inhibitors. One and the same growth factor may have different activities depending on the target cell type, lineage of differentiation and the conditions under which it is tested (17,238,390). This duality of biologic activity by growth factors on cell growth appears to be a widespread phenomenon. For example, anti-proliferative effects have been found induced by several growth factors, such as EGF on squamous carcinoma cells, IL-1 on breast cancer cells, bFGF on Ewing's sarcoma cells, even though these growth factors are almost always to stimulate growth in other types of cell (238,390). TGFβ has been viewed as a growth-stimulatory factor for mesenchymal cells,
whereas it is predominately considered as a negative growth regulator for a variety of epithelial or lymphoreticular normal cells (43,289,390). Understanding the mechanism of TGFβ inhibitory activities at molecular levels may be helpful for studies on other growth inhibitory growth factors or cytokines.

TGFβ was originally described as a "transforming" growth factor that induced normal rat kidney fibroblasts to proliferate in soft agar assays in the presence of EGF. Subsequently, it was shown that TGFβ exhibits a remarkable diversity of other biological activities in addition to its effects on cell proliferation, and is now known to belong to a large family of related molecules having a wide range of biologic activities (43,269,391). The three distinct molecular forms of TGFβ identified in mammals have been designated TGFβ1, TGFβ2 (also called BSC-1 cell growth inhibitor or polyergin) and TGFβ3 (289,324,391,425). These molecules are first synthesized as larger precursor polypeptides and then processed to yield 12.5 kDa monomers (43). The biologically active, mature 25 kDa polypeptide consists of two identical disulphate-linked monomers. The precursor of TGFβ, or the latent form, is not biologically active unless the molecule is cleaved, or activated by physicochemical or enzymatic treatment. Plasmin appears to be involved in the activation of latent form TGFβ (43).

Like other growth factors, TGFβs bind to plasma membrane receptors and trigger a subsequent intracellular signalling network, inducing various biologic responses. Analysis of the binding properties of TGFβs to cellular membranes suggested the presence of multiple forms of TGFβ receptors. By means of membrane receptor labelling assays, it was revealed that up to nine distinct proteins can bind TGFβs (269). The type I receptor is a cell surface
glycoprotein of 53 kDa while the type II receptor is about 75 kDa. Both are expressed by almost all non-neoplastic cells (51,72,269). Type I and type II receptors appear to have different binding affinities to TGF-β isoforms, which correspond to the inhibitory activities of TGFβ isoforms. However, the three isoforms of TGFβs have the same binding characteristics to the betaglycan receptor (also known as the type III receptor), which is a membrane-anchored proteoglycan that can bind TGFβ via the core protein (72,269). Recently, the cDNA sequences for the three TGFβ receptors have been cloned. From these sequences, it was predicted that both the type I and the type II receptors are serine/threonine kinases with a cysteine-rich extracellular domain and a predicted cytoplasmic serine/threonine kinase domain (105,243). The type I and the type II receptors appear to interact with each other directly, while functionally the type I receptor requires the type II receptor to bind specific ligands and the type II receptor requires the type I receptor in order to signal (10).

It seems that the type I receptor may be responsible for the effect on extracellular matrix and induction of junB expression, whereas the type II receptor is involved in growth inhibition through pRB (74). Different from the type I and the type II receptors, the type III TGFβ receptor has a short cytoplasmic region with no obvious signalling motif (248,448). In the type III receptor, the transmembrane and short cytoplasmic regions are highly identical to the corresponding regions of endoglin, a disulphate-linked dimer of 95kD subunits expressed in vascular endothelium (248,269,448).

TGFβ can inhibit the wave of DNA synthesis following stimulation of quiescent cells when added at any point prior to the G1/S boundary (3,289). It has been shown that TGFβ can reduce c-myc gene expression and protein production, which may, in some instances be
one of the central events in the growth inhibitory response mediated by TGFβ (55,289,327,347). Down regulation of c-myc by TGFβ seems to be at the transcriptional level since deletion studies indicated that a cis-acting regulatory element of human c-myc promoter is responsible for the suppression of c-myc (289). Transformation of skin keratinocytes with human papilloma virus (HPV) 16 or 18, adenovirus E1A, and SV40 large T antigen can block the effect of TGFβ on c-myc expression and on cell proliferation (89,280,289,324).

Besides the inhibitory effect on c-myc, TGFβ is now known to affect some other components of the cell cycle control machinery, such as the phosphorylation of pRB (74,121,327); pRB is highly involved in the regulation of several transcriptional factors and cell cycle control proteins (181,383). However, the question still remains as to whether pRB is the mediator of TGFβ-induced growth inhibition (234,289,315,345). G1 cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors have been found to be a target of TGFβ in terms of its growth regulatory functions. It has been shown that treatment with TGFβ can suppress cyclin A and/or cyclin E mRNA, and down-regulate cyclin A protein, in several cell types (10,181,386). The effect of TGFβ on CDKs appears to be at both translational and posttranslational levels. The enzymatic activities of several CDKs were also found to be inhibited following the treatment with TGFβ, such as CDK2 and CDK4 (10,181). Overexpression of CDK4 has been found in cancer cells and such overexpression render cells insensitive to growth arrest induced by TGFβ (181). Studies on TGFβ-induced negative growth regulation led the recent discovery and cloning of a CDK inhibitor, called p27Kip1 (329,330,386). TGFβ induced growth inhibition is associated with an increased gene expression and protein production not only of p27Kip1, but also of other CDK inhibitors, such
as p15, p16, and p21\textsuperscript{WAF1} (10,93,181,330). Thus, it is clear that during the TGFβ-induced growth inhibition process there are multiple biochemical and functional changes in both the \textit{yin} and the \textit{yang} sides of cell cycle control, which includes a decrease in gene expression and protein activities of cyclins and CDKs and an increase in CDK inhibitors. However, it still remains unclear whether such changes are the causes or the consequences of a growth arrest brought about by exposure to active TGFβ isoforms.

TGFβs inhibit the proliferation of almost all non-transformed epithelial cells in culture, including rapidly growing skin keratinocytes or quiescent keratinocytes stimulated by exogenous EGF. Such inhibitory effects on normal cells have been considered to be an important factor serving to regulate the growth state; cellular loss of responsiveness to growth repression mediated by TGFβ may be a contributing factor to uncontrolled tumor cell proliferation (55,113). A stepwise increase in resistance to TGFβ-induced growth inhibition has been found during tumor progression as cancer evolves to a more malignant phenotypes, eg. in human colorectal carcinomas and gliomas (192,265). Some cells which are resistant to TGFβ-induced growth inhibition appear to have no detectable the type I or the type II receptors, as assessed by ligand cross linking analysis (72,269), and affinity labelling and gene expression (201,261), such as in colon cancer cells (261) and human T-cell malignancies (201). In this case, responsiveness to TGFβ induced growth inhibition can be restored experimentally by introduction of a cDNA expressing the lost receptor, such as the type II receptor (187).

More recently, it has been found that in human colon cancer cell lines and other tumors there are mutations in the TGFβ type II receptor gene, which produces a non-
functional receptor thereby allowing such cells to escape TGFβ-induced growth inhibition (267). In primary tumors from patients with hereditary nonpolyposis colorectal cancer, there are mutations in the type II receptor gene in as many as 90% of the samples tested (319). Recently, gastric cancers have also been found to have a similar mutations in type II receptor gene (296). It has been shown that the inactive TGFβ receptors may act as a dominant negative inhibitor of TGFβ-dependent transcription (54). Therefore, it has been proposed that the normal TGFβ type II receptor can be considered to act as a tumor suppressor (296,319).

In addition to the defects in receptors, growth resistance can also be a result of "tolerance" to such factors. Endogenous production of TGFβ and growth resistance to exogenously added TGFβ have been found in some human cancers. In these cells, down-regulation of constitutive TGFβ production was found to result in an increase in cell proliferation and in tumorigenicity, which suggests a partial or complete resistance of TGFβ-induced growth inhibition (465). Thus, as with the development of cancer, resistance to TGFβ-induced growth inhibition may occur sequentially and increasingly via multiple steps during the development and progression of the disease (113).

iii): Paracrine and autocrine growth factor, mechanisms regulating tumor growth:

Growth modulators have been studied in considerable detail for their physiological role in the control of cell proliferation and differentiation, and their potential pathological contribution in the autonomous growth of cancer cells. Some growth modulators can travel from distant organs through the blood stream to target cells in an endocrine manner; such modulators are normally called hormones. Other growth factors and cytokines which affect
particular target cells may also be produced by nearby cells (paracrine growth factors) or by
the target cell itself (autocrine growth factors) (3,389,450). The interaction of growth factors,
cytokines and hormones with their specific receptors triggers a cascade of intracellular
biochemical signals, resulting in the activation or repression of various subsets of genes.
Malignant cells arise in part as a result of a stepwise progression of genetic events that include
the 'pathologic' or abnormal expression of growth factors, or the various components of their
signalling pathways (3).

The paracrine mechanisms of cell growth are important in both physiological and
pathological regulations. In the context of tumor progression and metastasis, paracrine
growth factors are numerous, complex and critical to many aspects of tumor growth and
spread. With respect to primary tumors, expansion and invasion into adjacent tissues can
occur, which obviously creates a new environment for the invading tumor cells. In addition
to the possible acquisition of "autonomous" growth characteristics, such tumor cells are
confronted with potential growth modulators either positive or negative, associated with the
new microenvironment. Depending upon their survival capability, some tumor cell may
flourish, while others may not. It has been shown that human melanoma cell lines that were
derived from patients of different clinical histopathological stages of disease progression have
a different, or even an opposite, response to the same growth factors (or cytokines) secreted
by dermal fibroblasts (88), or endothelial cells (335).

In addition, tumor cells can also secrete factors which can modulate adjacent normal
or stromal cells to facilitate their growth and expansion, e.g. angiogenesis growth factors. It
is known that progressive growth and expansion of solid tumors beyond 1-2 mm in diameter
requires an adequate blood supply for oxygen and nutrients to be delivered to tumor cells and for removal of catabolites. Thus, there is an increasing demand for and development of new blood vessels from existing vessels, a process called angiogenesis (112). There are a number of growth factors secreted by tumor cells which can directly, or indirectly, induce angiogenesis. One such factor, namely, vascular endothelial growth factor (VEGF), or vascular permeability factor (VPF), has been isolated and known to be primarily responsible for the growth stimulation, differentiation, survival and function of endothelial cells; it plays an important role in both physiological and pathological angiogenesis (11,104,226,295,376).

It has been shown that tumor cells of many types, as well as tumor-infiltrating lymphocytes, but not endothelial cells, can produce VEGF and secrete it into the microenvironment. There VEGF can act on the endothelial cells by binding to specific receptor tyrosine kinases expressed on activated endothelial cell surface to induce their growth, differentiation and ultimately to stimulate angiogenesis (104,119,226,339,376). Inhibition of VEGF activity, eg. by neutralizing antibodies, can inhibit tumor growth and metastasis in vivo (19). Down-regulation or upregulation of VEGF expression in tumor cells by gene transfection methods can result in a decrease or increase in tumor growth in experimental animals, respectively (85,356). These observation clearly indicate the important role of paracrine VEGF in angiogenesis and the growth of solid tumors.

In some cases, tumor cells express an increased number of a particular growth factor receptor and can show enhanced responses to the respective growth factors at low concentrations. For example, an increase in EGF receptor level has been found to be associated with a worse prognosis or enhanced metastasis in breast, ovarian, colon and
bladder carcinomas as well as melanomas; highly metastatic tumor cell populations often have higher number of receptors to various paracrine growth factors produced by target organs (308). Using experimental models of metastasis, it is been found that organ preference of metastasis is sometimes correlated with an enhanced mitogenic response to particular growth factors and cytokines that are released from target organs (308). Tissue-conditioned medium contains growth factor(s) that stimulate the growth of target-metastasizing tumor cells in a relatively specific manner. Some of the growth stimulatory factors have been purified and identified, eg the lung-derived growth factors and bone marrow derived growth factors; both are transferrin or transferrin-like proteins as discussed earlier (308).

Autocrine production of growth factors is a concept that emerged over 15 years ago and has been subjected to extensive analysis in the context of cancer research, especially in the oncogene field (389). Evidence has been found in many human tumor systems indicating an important role for autocrine production, secretion and utilization of growth factors in the development and progression of human cancers. Since cancer is a result of multiple independent molecular perturbations which accumulate to cause the uncontrolled growth of cells, autocrine production of growth stimulatory factors is only one of the essential elements in the tumorigenic process. For example, if immortalized but non-tumorigenic cells are genetically manipulated so that they can produce abundant levels of a particular autocrine growth factor, this in itself is not sufficient to cause tumorigenic competence. However, an increase or onset in autocrine growth factor production may contribute to the process and to an increase in malignant aggressiveness (102,235). Autocrine growth factors such as PDGF, EGF (TGFα), and FGFs have been studied in this context (75,235,389,474).
The mechanism of growth stimulation by the "ectopic" autocrine production of growth stimulatory factors in neoplastic cells has been proposed to have both "public" and "private" loops (56). In the public loop, growth factors produced have to be secreted from the cell into the surrounding environment. The secreted factors may then bind to appropriate membrane receptors expressed by the producing cell and trigger an intracellular signalling network. In this case, the biological activities of the growth factor can be substantially or even completely abrogated by use of neutralizing antibodies specific for the growth factor or its receptor. The secreted factor may also act on adjacent cells of various types in a paracrine manner (56).

The private or intracellular autocrine loop, is also referred to as an "intracrine" mechanism of cell growth. In this situation, secretion is not required for the growth factor to bind to the receptor in order to trigger signal transduction and cell growth. There are several lines of evidence to support the concept of such a private autocrine loop. The cotransportation of secreted protein and transmembrane protein have been observed in endoplasmic reticulum cisternae, Golgi compartments and secretory vesicles, implying the existence of a general mechanism for protein migration (56). Extracellular addition of neutralizing antibodies to the growth factor into the cell culture medium does not neutralize its biological activity. However, intracellular microinjection of the neutralizing antibody is capable of abrogating some of its biological activities in vitro (150). In addition, use of specific antisense oligonucleotides to a particular growth factor encoding gene or its receptor encoding gene in some circumstances can abrogate its biological activity in a specific manner (32,33). Examples of private autocrine loop include PDGF, IL-3 and bFGF (56,235).
With respect to autocrine growth factors, there is also an autocrine production of growth inhibitory factors, such as TGFβ (43,389). This type of autocrine growth factor may contribute to some extent to the maintenance of a quiescent state of cell growth. Although TGFβ is known to be a potent inhibitor of cell proliferation, it has been found that many types of cell, both normal and neoplastic, can produce this factor, and express TGFβ receptors as well (43,204,324,465). In addition to its growth inhibitory activities, TGFβs can also induce other cellular changes, such as induce the production of VEGF and PDGF, formation of an extracellular matrix, etc., which may ultimately be favourable for TGFβ-producing cells (43,289). The ability of tumor cell produced TGFβ to facilitate the growth of solid tumors, eg. by inducing angiogenesis and tumor stroma formation, would have to be preceded by acquisition of resistance to its direct antiproliferative effects.

iv): Growth factors associated with human cutaneous melanoma:

In the context of human cutaneous melanoma, the pathogenic role of growth factors, especially autocrine growth factors has also been studied in some detail. Among growth factors which stimulate cell growth, basic fibroblastic growth factor (bFGF) and melanoma growth stimulatory activity (MGSA) are biologically active in, and produced by, most if not all stages of melanoma disease progression, from benign nevi to metastatic melanoma. In contrast, they are not constitutively produced by normal human melanocytes (102,148,343,348,379).

The discovery of bFGF as a growth factor for normal melanocytes was a critical step in the search for growth regulators that are biologically relevant to melanocytes (148). The
functions of bFGF and other members of this family have been studied intensively and include stimulation of cell proliferation, differentiation and migration, through its binding to, and activation of, the receptors on target cells. It is considered that bFGF is one of the key players in angiogenesis, wound healing and tumor invasion and metastasis (3,89,136,202). bFGF is mitogenic to melanocytes in in vitro culture, and appears to be provided mostly by adjacent basal keratinocytes in situ (normal melanocytes do not produce bFGF on their own at detectable levels although their growth is stimulated by exogenously added bFGF (148)). The expression of bFGF receptors have been detected in normal melanocytes (148,348). Constitutive expression of bFGF has been found in dysplastic and dermal nevi, and in primary and metastatic melanomas (148,264,468). Induced down-regulation of bFGF production by antisense oligonucleotides or intracellular introduction of bFGF neutralizing antibodies was found to inhibit the proliferation of human melanoma cells, implicating an autocrine stimulatory role for bFGF in melanomas (33,150,206). Down-regulation of bFGF receptor expression by antisense oligonucleotides has also been shown to cause melanoma cell growth inhibition in vitro (32).

Another growth factor, known as MGSA, was originally described as an autostimulatory growth factor for the Hs294T human melanoma cell line. After biochemical purification of the protein and molecular cloning of the gene, it was found that MGSA is homologous to the gro gene product and is a member of the β-thromboglobulin superfamily, including interleukin-8 (IL-8) (46,343,344). The human MGSA gene has been mapped to a region of chromosome 4, where c-kit, the steel factor receptor is also located (58,344). The biologic activities of MGSA include growth stimulation in normal melanocytes, nevocytes,
and melanoma cells. Receptors for MGSA also present in all these melanocytic lineage associated cells. Interestingly, purified MGSA could compete with IL-8 for the binding to IL-8 receptor. Overexpression of MGSA in immortalized melanocytic (melan-A) cells by transfection of a cDNA vector expressing the MGSA gene resulted in a 'transformed' phenotype in one of the two MGSA expressing transfected clones, indicating a possible role for MGSA in melanocytic transformation (343). Stable expression of MGSA has been found in both benign nevi and malignant melanoma cells, which may be the result of constitutive activation of NF-kB (463).

Although autocrine production of bFGF and MGSA is a common feature of benign and malignant melanocytes, genetic manipulation of normal melanocytes so that they produce bFGF was not found to oncogenically transform the transfected melanocytes (102), suggesting additional requirement for other cellular and genetic changes, ie. the acquisition of autocrine production of such growth factors may be necessary but not sufficient for oncogenic cell transformation.

IL-8, a member of the same chemokine family as MGSA, was also found to be expressed in some human melanomas. Autocrine stimulatory activity has been reported in human malignant melanoma cells, and seems to be correlated with metastatic potential (384,385). Serum IL-8 levels are elevated in patients with metastatic melanomas (363).

In addition, TGFα has also been implicated in human melanoma cell as a possible autocrine growth stimulating regulator (61,348,379). Expression of TGFα transcripts and production of TGFα-like activity have been detected in human melanoma tissue specimens and in cell lines, but not in normal melanocytes. The EGF receptor that TGFα binds is highly
expressed in metastatic melanomas, and exogenously added EGF can stimulate the proliferation of some human melanoma cell lines (99,348).

Abnormal production of other growth regulators, such as PDGF, keratinocyte growth factor, NGF and IL-1 have also been observed in human melanoma cells. However, there is no definitive information yet about the role of these autocrine growth factors in regulating the proliferation of melanoma cells (7,343,348). Expression of PDGF has been found in about 50% of the cultured melanoma cells, but not in normal melanocytes. Both IL-1α and β are detectable at the mRNA level and protein levels. NGF is also detectable both in cultured cells and \textit{in situ} (33,348). Various combinations of these growth factors can be co-expressed in a given melanoma cell line.

In addition to the autocrine role of certain growth factors in stimulating the proliferation of human melanocytes and melanoma cells, growth modulators from endocrine and/or paracrine sources are also important. The best example is melanotropin (α-MSH) that comes from the pituitary gland and regulates the differentiation of melanocytes. Insulin and insulin-like growth factors are among the major factors that stimulate the proliferation of both normal melanocytes and malignant melanoma cells (343,348). Hepatocyte growth factor (HGF), also known as scatter factor (SF), or hepatopoietin A (or hepatotropin), is able to stimulate the proliferation of a wide variety of epithelial cells. The receptor for HGF has been identified to be the product of a proto-oncogene \textit{c-met}. Both normal melanocytes and malignant melanoma cells express the \textit{c-met} gene and protein, and proliferation of such cells is stimulated by exogenously added HGF (151).

In summary, there exists a large number of possible autocrine or paracrine growth
factors for human cutaneous melanoma cells. However much work needs to be done to evaluate the functional contributions of most of these factors to human melanoma biology, with respect to their growth both \textit{in vitro} and \textit{in vivo}, especially the latter. As it stands, the current literature in this area remains largely descriptive.

3. BACKGROUND OF THE PROJECT:

i): Normal, benign and malignant melanocytic cells: a tool to study human tumor progression.

Human melanocytic cells have been used widely as a model for studies on tumor progression because of the fact cells or cell lines can be cultured \textit{in vitro} from virtually all lesions along the tumor progression pathway, including normal melanocytes, atypical benign nevi, different stages of primary melanoma, as well as metastatic melanomas (149,205,466).

The first successful attempts to culture normal human melanocytes were reported by Eisinger and Marko in 1982. It was found that the addition of the tumor promoter phorbol 12-myristate-13-acetate (TPA) and cholera toxin to the culture medium was required to obtain sustained growth of cultures of pure melanocytes without overgrowth or contamination by other cell types (106). Since then considerable progress has been made in understanding human pigment cell biology at both the cellular and molecular levels. In culture, normal melanocytes require certain growth supplements in the base medium to maintain cell proliferation. The growth supplements include growth factors and hormones, such as EGF, transferrin, bFGF, insulin (or IGF-1) and \( \alpha \)-MSH, in addition to other agents i.e. the protein
kinase C activator, TPA (162,379,466). The life span of melanocytes in cell culture is generally limited to about 50 population doubling (162,379,466). Benign nevus cells from common acquired and congenital nevi have similar characteristics to melanocytes in terms of their requirement for growth supplements and expression of a limited life span in cell culture, with the exception that they require less bFGF and TPA for growth and survival (162,264,275,379). As already discussed, constitutive production of bFGF occurs in benign nevi, and this probably accounts for their decreased bFGF dependence.

Human melanoma cell lines have been successfully derived from tumors of patients at different clinical and histopathological stages of disease progression, primarily by Herlyn and coworkers, as well as by a few other groups (66,130,162,205). Owing to some technical difficulties, however, there are only a few cell lines that have been successfully established from very early-stage primary lesions, such as the radial growth phase (RGP), or 'thin' vertical growth phase (VGP) (<0.76mm in tumor thickness) (162,205). In contrast, virtually hundreds of melanoma cell lines have been established from advanced-stage tumors, both primary 'thick' VGP lesions and distant metastases, particularly the latter (205).

Human melanoma cell lines have been classified by Herlyn and his coworkers into three major groups depending upon the clinical and histopathological feature of the tumor that the cells were derived from, and on the basis of certain biochemical and immunological features of the cells. These three groups are referred to as "early", "intermediate" and "advanced" melanomas (205). The early-stage group, (including cell lines such as WM35, WM 1341B, WM793 and WM902B, which were used extensively for the studies described in this thesis), was derived either from RGP or from "thin" VGP primaries. These patients
have been followed up for 7 - 12 years without any sign of recurrence or metastasis after surgical removal of their primary tumors (205). The advanced-stage group refers to many human melanoma cell lines established from either thick VGP primary tumors in patients with simultaneous evidence of distant metastases or from distant metastatic lesions per se, (which include cell lines, such as WM983a, WM1361A, and WM 9 etc.) (9,143,205). The intermediate group is classified as cell lines derived from primary tumors that can be classified as early-stage group by some parameters but as advanced-stage by others. These patients had no evidence of metastases at the time their primary tumor were removed, but had metastases or second primary tumors at a later time. These tumors seem to undergo a transition from early-stage to advanced-stage, or they contain mixtures of tumor cells of both stages (9,143,205). Among this group there are several cell lines such as WM75, WM115, and WM278, which have derivative cell lines called WM 373, WM 165, and WM 1617, respectively. These latter cell lines were derived later from metastases or second primary tumors of the same respective patients.

Rigorous characterization of these human melanoma cell lines has been carried out in respect to their requirement of growth stimulatory factors to grow in vitro. These studies revealed that melanoma cell lines require much fewer growth supplements compared to normal melanocytes or benign nevus cells (162,205,379). Furthermore, advanced-stage cell lines can be adapted to grow in medium under serum free conditions whereas early-stage or intermediate stage cell lines fail to do so. This may be the result of endogenous production and utilization of a number of growth stimulatory factors by advanced-stage melanoma cells, such as bFGF, TGFα, TGFβ, PDGF, etc. (162,205,379). Such autocrine growth factor
stimulation provides the cells with an ability to grow independently of exogenous growth factors that are normally provided by normal host cells in vivo in their immediate microenvironment. Such so-called "autonomous" growth is a common feature of many advanced human cancers.

ii): Molecular and cellular markers of melanoma progression:

There are a large number of reports dealing with changes in gene expression and protein production detected in melanoma cell lines as a function of disease progression from normal, benign to malignant melanocytic cells. These studies deal with changes in expression of growth stimulatory mostly autocrine factors and their receptors, antigen expression, adhesion molecules, proteases, among many other traits (148,162,325,343,348,353,379,466). However, there were no published reports (at the time I began my thesis project) dealing with the possible involvement of inhibitory growth factors in the progression of human cutaneous melanomas.

At the time my thesis studies were initiated, probably the most important finding in the growth factor/melanoma field was the identification of bFGF as the major growth factor for melanocytic cells. It is also known that bFGF is produced by dermal fibroblasts, keratinocytes, and that its production is up-regulated by exposure to UV light; this may provide normal melanocytes in situ with the necessary supply of bFGF (148,379,466). On the other hand, bFGF is apparently an autocrine growth stimulator for both benign nevus cells and malignant melanoma cells (as discussed above) . Further analysis at the levels of gene expression and protein production indicated that advanced-stage melanoma cells produce a
number of growth regulatory factors that normal melanocytes or nevus cells do not. These include TGFα, IL-1, TGFβ, keratinocyte growth factor and NGF (7,62,225,343,348,349,379). An increased expression of various growth factor receptors is also detected (99). Furthermore, the expression of protein kinase C and cAMP-dependent protein kinase appeared to have different patterns when comparing normal and malignant melanocytic cells (31). Taken together, such studies clearly demonstrated an association of growth stimulatory factor production, biological function of such factors and progression of melanocytic tumors.

Recently, subtraction hybridization after treatment of melanoma cells with mezerein and interferon β, has been used to identify a number of genes that may regulate human melanoma differentiation, growth and progression (194). Among them, the melanoma differentiation associated genes (mda) 6 and mda 7 appear to have antiproliferative activity for cell growth and function as negative regulators during melanoma progression (194,195). Indeed, mda 6 has been identified as one of the universal cyclin-dependent kinase inhibitors, best known as p21, or CIP1, or WAF1 (135,194).

With respect to other molecular alterations, a recent report from this laboratory showed that there is a constitutive expression of 72-kDa gelatinase A in both early and advanced-stage melanoma cell lines, whereas only advanced-stage melanoma cells express 92-kDa gelatinase B (259). There is also a report showing that human metastatic melanoma cell lines expressed very high levels of ‘tissue factor’, the major cellular initiator of the plasma coagulation protease cascade, whereas normal melanocytes and an early-stage melanoma cell line WM35 expressed it only at low levels (291). Use of inhibitory antibodies to tissue factor
or inhibition of tissue factor receptor function was shown to reduce the growth of human metastatic melanoma cells in nude mice.

The involvement of integrin receptors in melanoma progression and regulation has also been evaluated. In particular there is an elevated expression of \( \alpha_v \beta_3 \) (the 'vitronectin receptor') in highly metastatic melanoma cell clones compared to low metastatic parental line of A375 (111,129). Studies from several groups have indicated that \( \alpha_5 \beta_1 \) (the laminin receptor) production gradually decreased, while the \( \alpha_3 \beta_1 \) integrin increased with tumor progression (299,300). The \( \alpha_5 \beta_1 \) integrin receptor appears responsible for the proliferative response of human melanoma cells in response to the mitogenic activity induced by fibronectin (288).

The role that other adhesion molecules may play in melanoma progression, especially in the transition from metastatically incompetent to metastatically competent stages, has also been studied by several groups. One such molecule is intercellular adhesion molecule 1 (ICAM-1). There have been reports which showed the expression of ICAM-1 in malignant melanoma cells and the existence of a significant correlation of ICAM-1 expression with progression stages of human melanoma (131,156,196,298). Expression of ICAM-1 was also shown to be up-regulated by addition of IL-6 in human melanocytes and melanoma cells (215). However, there are also conflicting reports indicating no correlation of ICAM-1 expression with metastatic melanoma potential (457); indeed even benign nevi lesions can express the ICAM-1 gene (98,155). A similar conflicting set of results was also reported in the analysis of VCAM-1 expression in human melanoma cells (98,197). When CD44, another adhesion molecule, which is a transmembrane hyaluronate receptor, was studied, results
showed that variant forms of CD44 are involved in tumor growth and the metastatic process (146,374,404). Human melanoma cells, deficient in CD44 expression, give rise to low number of lung metastases when injected in nude mice (40). Other studies have indicated that tumor progression or metastatic potential of human cutaneous melanomas may be associated with alterations in expression of certain antigens (143,353), expression of perlecan, a proteoglycan (83), or expression of calcyclin, a small calcium binding protein (460).

In summary, there is clearly a very large number of molecular changes which accompany the progression of human melanoma. Most of these changes involve upregulation in expression, while a smaller number is associated with a progressive decrease in expression. How any one of these particular changes contributes to progression and/or metastasis is, for the most part unclear.

iii): The differential effect of dermal fibroblasts on the growth of human melanoma cell lines derived from different stages of disease progression.

The participation of adjacent normal cells or tumor associated stromal cells in helping to regulate the proliferation and differentiation of cancerous cells has been known for years (116,365). Requirement of a blood supply in solid tumors to maintain their continuous growth and expansion is the most obvious example of this: tumor cells have to interact with endothelial cells to induce angiogenesis (116,244). During the multistep process of tumor invasion and metastasis, cancer cells also have to interact with basement membranes in order to break tissue compartment boundaries; they must also interact with other normal cells to gain access to, and remain in new sites (244,431,432). In this regard, there has been a number of studies which have shown that, depending upon the circumstances, the proliferative
properties of cancer cells can be negatively or positively regulated by adjacent normal cells, or their products (63,103,132,292,333,390,430).

Clinical observations have indicated that the vertical thickness of primary human cutaneous melanomas is the most reliable prognostic marker, i.e. increasing tumor thickness is clearly related to increasing eventual mortality (81). In addition to the thickness of tumors, the mitotic rate of tumor cells is also considered to be a useful predictive parameter of patient survival (81). RGP melanomas, which by definition are restricted to the epidermal compartment, have very little, if any, capability for sustained growth in the dermal mesenchyme. In contrast, thick VGP melanomas are able to proliferate in this "foreign" site, and this can obviously provide invading tumor cells with the access to the vascular and lymphatic systems, which may then generate distant metastases (81). Some tumor cells from metastatically-incompetent, early-stage melanomas might micro-invade into the dermal mesenchyme as single cells or small nests of cells, but these cells apparently do not actively proliferate to form an expansile tumor mass. This is a key phenotypic difference from metastatically-competent melanoma cells, which do invade into, proliferate and expand in the dermal mesenchyme (81,144). This difference, at least in part, could be related to a differential sensitivity of early vs advanced stage melanoma cells to inhibitory molecules released by normal cells in the dermal mesenchyme. An obvious hypothesis would be that early stage, metastatically-incompetent cells are sensitive to one or more paracrine growth inhibitors secreted by dermal mesenchyme-associated cells, whereas advanced-stage (metastatically-competent) cells are partially, or fully, resistant to these growth inhibitors.

To test this hypothesis, human dermal fibroblastic cells were chosen for further
analysis by Isabelle Cornil et al in Dr. Kerbel's laboratory (88). The reason for the choice of dermal fibroblastic cells is that this cell type is the most abundant in the dermal mesenchyme. In the experiments undertaken, normal dermal fibroblastic cells were isolated from neonatal foreskins and were co-cultured with established melanoma cell lines that were derived from either early- or advanced-stage lesions of disease progression. Cell growth was measured by either $[^3]H$-thymidine incorporation or direct cell counts. The results showed that fibroblasts caused a significant inhibition of melanoma cell growth in cell lines that were derived from early-stage lesions, (such as WM35 derived from a primary RGP, and WM902B and WM1341B which came from 'thin' VGP primary lesions). In contrast, under identical conditions, growth was stimulated when human melanoma cell lines established from advanced-stage lesions were tested; this was true using either tumors from advanced primary or metastatic lesions (88). This differential effect on early versus advanced stage melanoma cell lines was shown to be "specific" since use of endothelial cells or adipocytes did not reproduce the activities expressed by fibroblasts. Subsequently, an attempt was made to understand the possible mechanism, which were responsible for mediating the differential effects of fibroblasts on the growth of melanoma cells obtained from different stages of disease progression. For example, does it involve direct cell contact, extracellular matrix components, or the release of soluble growth factors? Direct cell-cell contact has been known to be essential for the function of some membrane-bound growth factors and intercellular communication (91). However, several lines of evidence indicated the soluble growth regulating factor(s) were involved in the results observed in the co-culture experiments (88). First, co-culture of melanoma cells with fibroblasts without direct cell-cell contact gave
same results. Second, addition of conditioned medium from fibroblast culture into cultured human melanoma cell lines mimicked the effects of fibroblast on melanoma cells. This indicated the possible release of a soluble growth factor by fibroblasts which behaved as an inhibitor or stimulator, depending upon the stage of tumor progression of the 'target' cells. Alternatively there might be a distinct inhibitor (biologically active only in early stage melanoma cells) and stimulator (biologically active only on advanced stage melanoma cells).

To further identify these putative molecule(s), specific neutralizing antibodies to several growth factors and cytokines, such as bFGF, TGFα, EGF, PDGF, IGF-I, TGFβ and interferon γ (all of which were known at the time to either stimulate or inhibit the growth of melanoma cells), were used to try and implicate one or more of these as the incriminating factors. However, use of such antibodies to these growth factors (or to their respective specific receptors) did not abrogate either the observed inhibitory or stimulatory activities of fibroblast conditioned medium on melanoma cells obtained from early or advanced disease, respectively (Cornil & Kerbel, unpublished observations).

iv) Summary: Goals of the thesis project.

From the above results it was concluded that the fibroblast-derived growth factors involved in the findings of Cornil et al. (88) might be new, and hence require biochemical purification to establish their identity. This task became the initial goal and focus of my project. The rationale was that the basis of clonal dominance of primary melanomas by metastatically-competent tumor cells might be better understood by identifying the nature of the relevant factor(s). In addition, a new class (or classes) of paracrine inhibitors of early
stage tumor cell growth might be uncovered. The same could be said with respect to growth stimulators of advanced stage (metastatic) cancer. Furthermore, such studies had the potential to reveal new insights into loss of negative growth control during tumor progression, and the ability of certain tumor-stromal cell interactions to facilitate the course of malignant tumor progression. Chapter 1 begins with a biochemical analysis of the nature of the fibroblast-derived growth factors implicated in the results of Cornil et al. These studies established the nature of the inhibitor as being identical to the cytokine known as interleukin-6. Chapter 2 deals with aspects of development of resistance to IL-6 inhibitory effect by advanced stage melanoma cells, and IL-6 receptor analysis in these cell lines. The transition of IL-6 mediated growth activity so that it becomes a stimulator of melanoma growth in vivo, in more advanced stages of tumor progression, is presented in Chapter 3. Studies summarized in Chapter 4, implicate another member of the IL-6 family - oncostatin M - as a growth inhibitor for human melanoma cells, especially in early stages of disease progression. This is followed by an overall Discussion section.
Chapter 1:

Interleukin-6: a Fibroblast-derived Growth Inhibitor of Human Melanoma Cells from Early but not Advanced Stages of Tumor Progression

1.1: ABSTRACT

Recently we reported that human dermal fibroblasts, or conditioned media obtained from such cells affect the growth of human melanoma cells as a direct function of tumor progression: melanoma cells obtained from early-stage (metastatically-incompetent) primary lesions were growth inhibited whereas cells obtained from more advanced (metastatically-competent) primary lesions, or metastases, were growth stimulated. Ion exchange and gel filtration chromatography of fibroblast conditioned medium revealed the inhibitor to be a protein of molecular mass between 20-30 kilodaltons and distinct from the stimulator. This is the approximate molecular weight of interleukin-6 (IL-6), a ubiquitous multifunctional cytokine known to affect in particular many kinds of haematopoietic and lymphoid cells. Since this cytokine is known to be made by fibroblasts we attempted to determine if the human fibroblast derived growth inhibitor (hFDGI) was identical to IL-6. Neutralizing antibodies specific for IL-6 completely eliminated the inhibitory activity of hFDGI. Moreover, exposure to human recombinant IL-6 was found to inhibit the growth of early-stage melanoma cells obtained from radial growth phase (RGP) or early vertical growth phase (VGP) primary lesions in 3 of 4 cases. In contrast, melanoma cells from a number of more advanced VGP primary lesions, or from distant metastases, were completely resistant to this IL-6 mediated growth inhibition. Acquisition of an 'IL-6 resistant' phenotype by metastatically-competent melanoma cell variants may provide such cells with a proliferative advantage within the dermal mesenchyme (a hallmark of melanoma cells which are malignant) helping them eventually to dominate advanced primary lesions, and to establish secondary growths elsewhere.
1.2: INTRODUCTION

The growth and spread of cancers can be strongly influenced by surrounding normal tissues in a variety of ways. Tumor angiogenesis, i.e. the absolute requirement of new blood vessel capillaries for solid tumors to grow beyond 1-2 mm in diameter, is perhaps the most striking illustration of this interaction (116). Similarly, a variety of different hormones or locally-produced growth factors and cytokines secreted by various normal cells, can stimulate tumor growth (3,307). In addition to causing such mitogenic effects, surrounding normal cells can, in other circumstances, significantly suppress the growth of tumors (103); this is especially evident in the case of an interspersed minority subpopulation of tumor cells surrounded by an excess of normal cells, such as fibroblasts (221).

With few exceptions the effects of a given normal cell population on the growth and behaviour of a particular type of tumor have not been studied in the context of different stages of tumor progression. It is possible, for example, that the effect of normal adjacent cells on the growth of tumor cells from primary lesions early in tumor progression is quite different from those of more advanced (metastatically-competent) primary lesions, or metastases. We recently uncovered an interesting example of this in the context of human malignant melanoma (88). This type of cancer is one of the few where lesional steps in tumor progression are well defined and readily detectable (81,160). Thus, most primary melanomas initially arise in the epidermis as horizontal plaque-like lesions -- the so-called 'radial growth phase' (RGP) primary melanoma (81). Despite invasion into the dermis, these are always curable by surgery and are thought to be comprised of poorly proliferating tumor cells incompetent for metastasis (81). The next stage is the vertical growth phase (VGP) which
can grow as an expansile nodule in the dermal mesenchyme (81,84,144). This step initially encompasses lesions with little competence for metastasis (usually those with a thickness <0.76 mm, a low mitotic count, and a brisk lymphocytic infiltrate) (81) and subsequently, those likely to acquire metastatic competence (e.g. thick lesions with a high mitotic count and absent infiltrating lymphocytes) (81). The latter generally have phenotypic markers associated with metastatic melanoma cells e.g. up-regulation of intracellular adhesion molecule-1 and the β3 integrin subunit (6,174,196). The final stage of melanoma progression is distant metastasis formation. We found that co-culture of human melanoma cell lines with irradiated human dermal fibroblasts affected the growth of the melanoma cells as a direct function of tumor progression: RGP or early-stage VGP primary tumor-derived cell lines were growth inhibited whereas a majority of more advanced VGP primary or metastasis-derived cell lines were growth stimulated under the same conditions (88). These results could be reproduced using fibroblast cell culture conditioned media thus implicating the involvement of one or more secreted paracrine growth factors (88).

From these results we speculated that either a single growth factor may be involved which behaves as an inhibitor for early-stage melanoma cells, but as a mitogenic agent for more advanced stage melanoma cells. Alternatively, two different factors may be involved — one being an inhibitor, active only on early stage melanoma cells, and a stimulator, active only on the more advanced stage melanoma cells. Either possibility would provide metastatically-competent melanoma cells with a relative growth advantage. In preliminary experiments we were unable to suppress the inhibitory or stimulatory influences of dermal fibroblasts on melanoma cell proliferation by addition of neutralizing antibodies to a number of known
growth factors thought to be involved in the growth of solid tumors. These included
transforming growth factor-beta (TGF-β), interferon α/β and interferon gamma, basic
fibroblast growth factor (bFGF), platelet-derived growth factor, insulin-like growth factor-1,
epidermal growth factor, or nerve growth factor (Cornil and Kerbel, unpublished
observations). We therefore decided to attempt to identify the nature of the growth factors(s)
involved by means of biochemical purification.

The purpose of the present report is to summarize our findings; they strongly implicate
IL-6, a 'haematopoietic' multifunctional cytokine, as the fibroblast derived growth inhibitor
for early-stage melanoma cells. This raises the intriguing possibility that IL-6 could be a
potential growth regulator of other types of non-haematopoietic human solid tumor,
depending upon stage of disease progression.

1.3: MATERIALS AND METHODS

i) Partial purification of human fibroblast-derived growth inhibitor (hFDGI) for
early-stage melanoma cells

A three-step purification procedure was employed involving Q-Sepharose anion
exchange chromatography, SP-cation exchange chromatography, and gel filtration
chromatography, using a Waters 650E HPLC Protein Purification System.

Human neonatal foreskin fibroblasts (5 to 20 passages) were cultured in Dulbecco's
modified Eagle's medium (Gibco, Grand Island, N.Y.) containing 5% fetal bovine serum
(FBS, Gibco) until completely confluent. After being washed twice with PBS, fibroblasts
were cultured in serum free medium W489 (MCDB 153 : L-15 = 4:1, Irvine Scientific, Santa
Ana, C.A., USA) at 37°C for 48 hours. Conditioned medium (CM) was then collected and concentrated using Amicon YM1 membranes (Danvers, M.A.). Concentrated CM was dialysed against 20 mM Tris buffer, pH 8.5 and loaded onto a Q-Sepharose column (2.5 x 8.5 cm) at a flow rate of 45 ml per hour. Bound fractions were eluted by KCl linear gradient (0.0 - 0.6 M) for 300 ml and then a step gradient to 2 M of KCl. Pooled fractions with high hFDGI activities (as assayed by inhibition of growth of WM 35 melanoma cells) from Q-Sepharose chromatography were dialysed against 25 mM glycine-HCl buffer, pH 2.5 and then loaded onto a Protein-Pak™ SP 8HR column (1.0 x 10 cm, Waters) at a flow rate of 60 ml per hour in the same buffer. After being washed in 70 ml buffer bound fractions were eluted first with KCl (0.0 - 0.3 M) and then with NaCl (0.0 - 1.0 M) linear gradients. Fractions containing high hFDGI activities from SP column were concentrated to about 0.7 ml using the Centriprep-10 (Amicon) and then injected into a Superose 12 FPLC column (16 x 515 mm, Pharmacia) in 25 mM Tris, pH 7.2 and 0.1 M NaCl at a flow rate of 30 ml per hour. One ml was collected for each fraction. Protein standards used were phosphorylase B (97.4 kD), bovine serum albumin (BSA, 66 kD), ovalbumin (45 kD), soybean trypsin inhibitor (21.5 kD), and myoglobin (17 kD).

ii) Cell Proliferation Assays:

These were done using [3H]-thymidine incorporation, essentially as described previously (88). To monitor hFDGI activity during purification, appropriate amounts (50 to 200 µl) of concentrated fibroblast-derived CM, or of fractions from the chromatographic studies, were added to dialysis tubing with 1 mg of BSA and dialysed against 20 mM Tris buffer (pH 7.4) and subsequently against serum-free W489 medium. They were then
sterilized through a 0.22 μm filter, and hFDGI bioactivity was assayed using an early-stage (RGP) human melanoma cell line, WM35, as a growth inhibition sensitive 'target.' Human melanoma cells (5,000 cells/well) were plated in 96-well plates and incubated with either hFDGI or cytokines for 36 to 48 hours. Cells were pulse labelled with [³H]-thymidine for 4 to 6 hours before being harvested.

To assess the effects of cytokines on melanoma cell growth, human recombinant preparations (Upstate Biotechnology Inc., Lake Placid, N.Y.) at various concentrations were added into cell culture in ExCell 300 medium and 1% FBS. Cytokines and their concentrations used were IL-1 α (0.5 pg - 50 ng/ml, 1 unit = 1 pg/ml), IL-1 β (0.05 - 50.0 ng/ml), 1 unit = 0.1 ng/ml), IL-6 (0.025 -250 ng/ml, 1 unit = 0.26 ng/ml) and tumor necrosis factor α (TNF α, 0.025 -250 ng/ml, 2 x 10⁴ units/mg). Highly specific polyclonal neutralizing antibodies to IL-6 (R & D Systems, Minneapolis, MN) were used as described in Results. All experiments were repeated at least twice and conducted by triplicate determinations. Controls without added factors were considered as 100%

iii) Cell Lines:

The origin of the melanoma cell lines used in these studies have been described in detail elsewhere by us (81) and by Herlyn and colleagues (160,162). Some details are also summarized in Table 2 of the Results section. The WM series of cell lines were kindly provided by Dr. Meenhard Herlyn of the Wistar Institute for Anatomy and Cell Biology, Philadelphia. Melanoma cell lines derived from RGP or early VGP primary lesions in which patients had no recurrence of disease (even 8-10 years later) are classified as 'early-stage'
Figure 1.1: Inhibitory activities of human fibroblast-derived growth inhibitor (hFDGI) on \(^3\text{H}\)-thymidine incorporation of WM 35 melanoma cells after (A) Q-Sepharose anion exchange chromatography, (B) SP cation exchange chromatography and (C) FPLC gel filtration chromatography. Numbers on (C) indicates molecular weight (K\(\Omega\)a).
lesions. Those derived from distant metastases, or from VGP primaries in which the patient had evidence of distant metastases, are designated as 'advanced-stage' lesions (160).

1.4: RESULTS

i) The human fibroblast derived growth inhibitor for early-stage melanoma cells is a protein of molecular mass between 20-30 kDa

Initially we attempted the isolation and purification of both the putative growth stimulator for advanced melanoma cells and the inhibitor for early-stage melanoma cells. Preliminary experiments showed that the stimulator (as assessed using the advanced-stage WM 9 melanoma cell line as the 'target') was not stable and could be separated from the inhibitor. Therefore, we decided to try to identify the growth inhibitor (i.e. human fibroblast-derived growth inhibitor, or hFDGI) of early stage human melanomas using the early-stage WM 35 cell line to monitor the biological (i.e. growth inhibitory) activity during the various biochemical purification steps. As summarized in Figure 1.1 and Table 1.1, the three-step purification gave 0.5% total protein recovery and showed that hFDGI has a molecular mass of 20 to 30 KDa. The relative broad hFDGI activity peak in FPLC gel filtration (Fig. 1.1C) was reproducible by concentrated CM indicating that the broad peak was not the result of acidic treatment of hFDGI in SP chromatography (data not shown). The semi-purified material of hFDGI after FPLC was then tested on other human melanoma cell lines. It will be noted (Fig. 1.2) that this semi-purified material retained inhibitory activity against three of four early-stage melanoma cell lines (the exception being WM 793) whereas no effect was observed on the growth of a large number of more advanced stage (metastatically-competent)
melanoma cell lines, whether these were derived from VGP primary tumors or metastases. It also suppressed the growth of a melanoma cell line called WM 75 (data not shown), similar to dermal fibroblasts (88). WM 75 behaves as a relatively early stage VGP primary melanoma (D. Guerry, personal communication). In a previous paper we had mistakenly designated this

Table 1.1. Summary of Purification of Human 'Fibroblast-derived Growth Inhibitor' (hFDGI)

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg) *</th>
<th>Protein Recovery (%)</th>
<th>Specific Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned medium</td>
<td>80.0</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>Anion exchange</td>
<td>14.2</td>
<td>17.7</td>
<td>128</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>2.0</td>
<td>2.5</td>
<td>1000</td>
</tr>
<tr>
<td>Superose 12 FPLC</td>
<td>0.4</td>
<td>0.5</td>
<td>2632</td>
</tr>
</tbody>
</table>

* Protein measurements were conducted by the enhanced protocol at 60°C as described by the reagent supplier (Pierce, Rockford, Illinois, USA). BSA was used as a protein standard and was obtained from the supplier.

** Specific activity is expressed as units per mg of total protein. One unit is defined as the amount of immobilized protein (mg) in 150 μl of culture medium which inhibited the proliferation of WM 35 cells by 50%.
cell line which was called WM 75c as having been derived from an advanced stage metastasis (88). The lack of any stimulatory activity on the advanced stage cell lines implied the existence of a growth stimulator distinct from the inhibitor, which was lost during the chromatographic separations. At the present time we have no further information on the nature of this growth stimulator. We also found that the inhibitory activity of hFDGI was completely reversible. Thus removal of hFDGI from cultures of WM 35 cells resulted in recovery from inhibition within 24 hours (Lu and Kerbel, unpublished observations).

**ii) Association of identity of hFDGI to IL-6:**

At this point several considerations prompted us to determine whether the inhibitory activity might be related to a particular member of the 'haematopoietic' growth factor/cytokine family, namely IL-6. First, it is known that the molecular weight of IL-6 (19-30 kD) is clearly in the same range as hFDGI, and it is also made by fibroblasts (217,434). The broad range in molecular weights is due to differential glycosylation of the polypeptide (217,434). Second, IL-6 has recently been shown (403) to inhibit the proliferation of normal human melanocytes (melanoma cells were not tested). Figure 1.3 and Table 1.2 summarize the evidence which demonstrate that the fibroblast-derived inhibitory factor for early-stage melanoma cells is indeed most likely identical to IL-6. This evidence can be summarized as follows: (i) the addition of specific neutralizing antibodies to IL-6 completely abrogated the activity of hFDGI against the early-stage melanoma cell lines such as WM 35 (Fig. 1.3A); (ii) as shown in Table 1.2 and Figure 1.3B, addition of human recombinant IL-6 to human melanoma cell line cultures resulted in growth inhibition of three of four early-stage melanoma cell lines, the exception being WM 793; WM 793 is likewise not growth inhibited
Figure 1.2: hFDGI inhibiting activities on human melanoma cell lines obtained from different stages of tumor progression. Five thousand melanoma cells of each cell line were incubated without or with partially purified hFDGI obtained after FPLC. Data are expressed as mean (±SD) from triplicate determinations compared with the controls of each cell line (considered as 100%). See also Table 1.2.
by addition of hFDGI (Fig. 1.2) or by coculture with irradiated fibroblasts (88). WM 75 cells were also inhibited (data not shown). In striking contrast, all of the more advanced stage-derived melanoma cell lines tested were found to be completely resistant to the inhibitory effects of exogenous IL-6 (Table 1.2 and Fig. 1.3B), even at doses of 250ng/ml where some stimulation was observed (Fig. 1.3B). It will be noted that IL-6 appears to be a very potent growth inhibitor (when it does inhibit) with concentrations in the 10-25 ng/ml range causing >90% inhibition of DNA synthesis. It is also noteworthy that, with the exception of tumor necrosis factor (TNF), there was a distinct trend towards sensitivity to the growth inhibitory effects of several cytokines manifested by early-stage melanoma cell lines, which was lost in the more advanced stage derived cell lines (Table 1.2). Thus, two of four of the early-stage cell lines (WM 35 and WM 793) were inhibited by IL-1α and IL-1β whereas none of the advanced stage cell lines was growth inhibited. A similar trend towards increasing resistance with melanoma progression to exogenously added transforming growth factor-beta (TGF-β) has also been noted by us (260). Thus acquisition of resistance to a number of structurally diverse polypeptide growth inhibitors seems to be a feature of advanced stage melanomas; this resistance is most consistently seen with IL-6.

1.5: DISCUSSION

Interleukin-6 is generally viewed as a member of the haematopoietic growth factor/cytokine family (217,434). In terms of its capacity as a potential regulator of tumor cell growth, most studies have emphasized its possible role as a paracrine or autocrine stimulatory growth factor for various types of haematopoietic malignancies (168) such as
Figure 1.3: (A) Neutralization of hFDGI activity by anti-hIL-6 antibody. 5,000 human melanoma cells were incubated with hIL-6 (12.5 ng/ml) or anti-human IL-6 (10 μg/ml) or hFDGI, or the combinations as indicated for 48 hours. Cells were then pulse labelled with [³H]-thymidine. 'ab' stands for 'antibody'. (B) IL-6 dose response curves on four independent human melanoma cell lines, MeWo and WM 9 (obtained from advanced stage metastatic disease), and WM 35 and WM 902B (obtained from early-stage metastatically-incompetent primary lesions).
multiple myeloma (240). It might therefore be expected that IL-6 should have little effect on the growth of non-haematopoietic solid tumors. Indeed a recent study by Serve et al. would appear to support this view as 27 different cell lines obtained from 12 different types of human cancer (mostly carcinomas) were found to be insensitive to exogenously added IL-6 (377). Our results, however, suggest otherwise, with the proviso that stage of disease be taken into consideration when assessing the possible influence of IL-6 on tumor cell growth. Thus the majority of melanoma cell lines from early-stage (metastatically incompetent) stages of tumor progression were found to be highly sensitive to IL-6 mediated growth inhibition. This sensitivity was completely lost, however, in all the advanced stage (metastatically-competent) melanomas that were examined.

The latter results (i.e the results with the metastatic cell lines) are consistent with the results of Serve et al (377) in that these authors used advanced stage cancers. However, clearly, it cannot be concluded necessarily that IL-6 is an 'inert' growth factor in the context of melanoma, and perhaps other types of solid tumor as well. This underscores the necessity of evaluating the response of tumor cells obtained from different stages of disease progression to a given growth factor when trying to determine the overall effect that factor has on tumor cell growth and behaviour. Unfortunately this has been ignored in most growth factor studies in human cancer, in part because it is frequently not possible to obtain early-stage lesions in most types of cancer, as it is in melanoma or colorectal carcinoma. The few studies that have been undertaken with this consideration in mind tend to support the view that increasing resistance to negative growth regulators such as TGF-β occurs with tumor progression (192,368,420). Since the tumor cells in many solid cancers have been found to express
Table 1.2. Effect of Cytokines on \(^{3}H\)-thymidine Incorporation of Human Melanoma Cells as a Function of Tumor Progression

<table>
<thead>
<tr>
<th>Cell line tested</th>
<th>Origin</th>
<th>Cytokines Tested:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-1α</td>
</tr>
<tr>
<td>WM 35</td>
<td>early-stage RGP (primary)</td>
<td>I (0.11) (^1)</td>
</tr>
<tr>
<td>WM 902B</td>
<td>early-stage VGP (primary)</td>
<td>NR (^2)</td>
</tr>
<tr>
<td>WM 1341B</td>
<td>early-stage VGP (primary)</td>
<td>NR</td>
</tr>
<tr>
<td>WM 793</td>
<td>early-stage VGP (primary)</td>
<td>I (0.5)</td>
</tr>
<tr>
<td>WM 1361A</td>
<td>advanced-stage VGP (primary)</td>
<td>NR</td>
</tr>
<tr>
<td>WM 983A</td>
<td>advanced-stage VGP (primary)</td>
<td>NR</td>
</tr>
<tr>
<td>WM 1205</td>
<td>metastasis</td>
<td>NR</td>
</tr>
<tr>
<td>WM 9</td>
<td>metastasis</td>
<td>NR</td>
</tr>
<tr>
<td>MeWo</td>
<td>metastasis</td>
<td>NR</td>
</tr>
<tr>
<td>WM 451</td>
<td>metastasis</td>
<td>NR</td>
</tr>
<tr>
<td>SKMEL 28</td>
<td>metastasis</td>
<td>NR</td>
</tr>
</tbody>
</table>
Legend, Table 1.2

1. "I" shows an inhibition in [³H]-thymidine incorporation of melanoma cells compared to controls (100%). Numbers in brackets are doses of cytokines (ng/ml) that caused 50% inhibition of [³H]-thymidine incorporation in melanoma cells (ID₅₀).

2. "NR" means no response (i.e. no inhibition or stimulation) and NT means not tested.

3. "S" indicates a slight stimulation (~150%) of [³H]-thymidine incorporation compared to controls (100%).

IL-6 activity in situ (405) this suggests that in some cases IL-6 may not only cease being a paracrine growth inhibitor but may actually behave as an 'ectopic' stimulatory autocrine growth factor (277). In this regard experiments are underway to evaluate the possible relationship of IL-6 production and IL-6 responsiveness in the melanoma cell lines studied in this report. Preliminary evidence indicates that cells from a number of advanced stage melanoma cell lines do indeed make IL-6 and that it functions as an autocrine growth stimulator in these cells (251). We have also recently undertaken Scatchard analyses of IL-6 receptor numbers and affinities in the melanoma cell lines studied here. No consistent differences were detected between early and advanced stage derived cell lines which could explain their disparate responses to IL-6 (251).

How might acquisition of an IL-6 resistant phenotype affect the course of melanoma
cell growth, progression and metastasis? Loss of sensitivity to an inhibitor such as IL-6 represents a form of 'progressive emancipation' from an external negative growth control (221) and this may endow rare metastatically competent melanoma cell variants with a growth advantage thereby helping them eventually to overgrow their non-metastatic counterparts within the primary tumor site (211,214). We have termed this process 'clonal dominance' of primary tumors by metastatically-competent tumor cell variants (211,214) a process which may enhance the probability of formation of distant metastases (421). Human melanoma appears to conform to this type of evolutionary growth pattern (6,81,162,174,211). Given the cellular ubiquity of IL-6 — e.g. it is produced by keratinocytes, endothelial cells, fibroblasts, macrophages, and monocytes (217,434) — it has considerable potential to facilitate the metastatic cell subpopulation clonal dominance process at the primary site, by impeding the relative growth of metastatically-incompetent tumor cells. This may be particularly true in the dermal mesenchyme where early-stage primary melanoma cells appear to have limited ability to proliferate unlike their metastatically-competent counterparts (81). This process may be facilitated by the fact that human melanoma cells can release IL-1 (36,225) which is a major inducer of IL-6 expression (217,434). Thus there is the possibility of a closed paracrine IL-6/IL-1 loop involving host cells, such as endothelial cells or fibroblasts, and melanoma cells, which could limit the proliferative capacity of metastatically-incompetent (i.e. IL-6 responsive) melanoma cells, both in the epidermis and the dermis.

Finally, we would point out that acquisition of resistance to the inhibitory effects of IL-6 by metastatically-competent cancer cells may be accompanied by resistance to a number of other potential growth inhibitors, such as IL-1α, IL-1β and TGF-β. This 'multi-growth
inhibitor resistant phenotype may be crucial to allowing metastatically-competent melanoma cells to proliferate readily in the foreign environment of the dermal mesenchyme (where blood vessel capillaries are located and to which they can gain access) and perhaps distant organ mesenchyme as well. These considerations also raise the intriguing notion that — conceptually similar to the phenomenon of multidrug resistance in cancer (53) — acquisition of resistance to one particular growth factor e.g. TGF-β, may lead to expression of resistance to other structurally unrelated growth factors, such as IL-6.

ACKNOWLEDGEMENTS

We sincerely thank Dr. Meenhard Herlyn for making the 'WM' series of human melanoma cell lines. Mr. M.F. Vickers was a summer student and helped in some of the cell culture work.
Chapter 2:

Interleukin-6 (IL-6) undergoes Transition From Paracrine Growth Inhibitor to Autocrine Stimulator during Human Melanoma Progression

2.1: ABSTRACT:

The ability to penetrate the dermal basement membrane and subsequently proliferate in the underlying mesenchyme is one of the key steps in malignant progression of human melanomas. We previously undertook studies aimed at assessing how normal dermal fibroblasts (one of the main cellular components of mesenchyme) may affect the growth of human melanoma cells and facilitate the overgrowth of malignant subpopulations (88). We found that melanoma cell lines from early-stage (metastatically-incompetent) lesions were growth inhibited whereas those from advanced-stage (metastatically-competent) lesions were stimulated under the same conditions by co-culture with fibroblasts; conditioned medium from such cells gave the same result. Subsequent studies using biochemical purification and neutralizing antibodies revealed the inhibitory activity to be identical to interleukin-6 (IL-6). We now report that addition of purified recombinant human IL-6 resulted in a growth inhibition in vitro by G1/G0 arrest of early, but not advanced stage melanoma cells. Despite this alteration in response there was no significant difference in melanoma cell lines of varying malignancy in respect to their expression of genes encoding the IL-6 receptor, or gp130, the IL-6 signal transducer. Scatchard analysis also revealed similar [125I]IL-6 binding activities in both IL-6 sensitive and resistant groups. However, studies of IL-6 production indicated that five out of eight IL-6 melanoma cell lines known to be resistant to exogenous IL-6-mediated growth inhibition constitutively expressed mRNA for IL-6; they also secreted bioactive IL-6 into culture medium. To assess the possible role of this endogenous IL-6 in melanoma cell growth, antisense oligonucleotides to the IL-6 gene were added to cultures of melanoma cells. This resulted in a significant growth inhibition only in cell lines that produced
endogenous IL-6. In contrast, neutralizing antibodies to IL-6 were ineffective in causing such growth inhibition. This indicates that endogenous IL-6 may behave as a growth stimulator by an intracellular ('private') autocrine mechanism. Thus, a single cytokine, IL-6, can switch from behaving as a paracrine growth inhibitor to an autocrine growth stimulator within the same cell lineage during malignant tumor progression. Such a switch may contribute to the growth advantage of metastatically-competent melanoma cells at the primary or distant organ sites and thereby facilitate progression of disease.

2.2: INTRODUCTION

One of the mechanisms by which cancer cells are thought to acquire a growth advantage is through the 'autocrine' production of mitogenic growth factors (3,389). This refers to the process whereby cancer cells, or their dysplastic cellular precursors, begin to express a growth factor (or receptor for the growth factor) which their normal cellular counterpart does not normally express. The receptors may be cell surface associated or expressed intracellularly, thereby creating 'public' or 'private' autocrine loops, respectively (56).

In the case of solid tumors, whether carcinomas, sarcomas, or tumors of neuroectodermal origin (e.g. brain tumors, malignant melanoma), the nature of many of these autocrine growth factors involved in their growth is well known (3). They include, for example, members of the fibroblast growth factor (FGF) family such as basic FGF (bFGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor, (EGF) and the insulin-like growth factors, IGF-I and IGF-II. Conspicuously
absent from this list are members of the haematopoietic family of cytokines and growth factors (134,281). These include the interleukins (ILs), colony stimulating factors (CSFs), erythropoietin and stem cell factor (SCF). Not surprisingly, these factors have been studied predominantly as paracrine or autocrine regulators of the growth of leukemias and lymphomas (3,134). However, there is a small (but growing) literature to suggest that members of the haematopoietic growth factor family may also help regulate the growth of solid tumors directly, especially in a paracrine manner (73,96,311,372). Prominent among these factors may be interleukin-6 (IL-6).

IL-6 is produced by a variety of cells including fibroblasts, endothelial cells, keratinocytes (all of which make this a potentially interesting cytokine for skin cancers, including melanoma), T and B lymphocytes and monocytes/macrophages (168,217,434). It is thought to be a major molecular mediator of inflammatory conditions, acute phase protein synthesis in the liver, and various immunological reactions (217). With respect to hematologic cancers it has been shown, for example, to behave as an autocrine growth factor for some lymphomas and human multiple myelomas in culture (208,240,316,475).

We recently uncovered evidence to show IL-6 can affect the growth properties of human melanomas differentially as a function of tumor progression (255). Thus, the growth of cell lines established from early-stage benign (curable) primary melanomas was inhibited by co-culture with normal dermal fibroblast or conditioned media from such cells (88). In contrast, cell lines established from more advanced (malignant) primary melanomas, or metastases, were stimulated under the same conditions (88). The activity responsible for the growth inhibition of early-stage melanoma cells was subsequently identified as being IL-6
the nature of the stimulator has not yet been determined. Consistent with these conclusions was the finding that addition of nanogram amounts of recombinant human IL-6 to early-stage melanoma cells was profoundly inhibitory to their growth whereas cells from more advanced cell lines were completely resistant to this inhibition (255).

The purpose of the present studies was to investigate the possible reasons for the transition in responsiveness to IL-6 during melanoma progression. Such a transition could provide malignant (i.e. metastatically-competent) melanoma cells with a growth advantage, helping them to acquire the ability to proliferate in the foreign environment of the dermal mesenchyme (81), and thus achieve 'clonal dominance' (211). We examined the IL-6 receptor status, and IL-6 production itself, among different melanoma cell lines obtained from different stages of disease progression. We found evidence that IL-6 is produced by over 50% of the advanced stage cell lines, and surprisingly, that it can function as an intracellular autocrine growth factor in such cells. Thus a single growth factor can change from functioning as a (paracrine) inhibitor to an autocrine stimulator within the same cell lineage during the multi-step process of malignant tumor progression.

2.3: MATERIALS AND METHODS:

Cell Culture and Growth Assay:

Human melanoma cell lines used and cell cultures were essentially the same as described in Chapter 1 in RPMI medium containing 5% FBS (88,160,162,255). In experiments to assess cell proliferation by [3H]thymidine incorporation rate, 5x10^5 cells were plated into 96-well plates with or without recombinant human IL-6 (rhIL-6) in triplicate
determinations in ExCell 300 medium and 1% FBS. Cells were cultured at 37°C for two days. Fifty μl of 2.0 μCi [³H]thymidine (25 Ci/mmol, Amersham) was added into culture wells and incubated for another 4 - 6 hours before cells were harvested in Titertek Cell Harvester 530 with Printed Filtermat A (Pharmacia). Radioactivity in the harvested filters was counted in 1205 Betaplate™ (Wallac-Pharmacia).

Flow Cytometric DNA Analysis:

Human melanoma cells (1 x 10⁶ cells) were plated in 100 mm dishes in RPMI medium containing 5% FBS overnight. Culture medium was then changed to RPMI medium containing 2% FBS without or with human recombinant IL-6 at 20 ng/ml concentration for 24 hours. Cells were harvested by trypsinization and washed three times with PBS. Cell pellet was then frozen and nuclei were prepared as described (440,441). DNA content was measured in an EPICS Elite Flowcytometer (Coulter Electronics Inc., Hialeah, FL) and data were analyzed with the program provided by the supplier.

Scatchard Analysis:

Determination of the affinities and sites of IL-6 binding to human melanoma cell lines was conducted essentially as described before by Taga et al. (406). Briefly, purified carrier-free rhIL-6 (Mw. 20.3 Kd, purchased from R & D Systems, Minneapolis, MN) was radioiodinated with Bolton-Hunter reagent (Amersham Cor., Arlington Heights, IL). [¹²⁵I]IL-6 was separated from free iodine in Sephadex G-50 column. Specific activity of [¹²⁵I]IL-6 was 4.4 x 10¹³ cpm/g as determined in a human myelomonocytic leukemia cell line, CESS (American Type Culture Collection, Rockville, MD) by self-displacement analysis. Incubation of [¹²⁵I]IL-6 with human melanoma cells (0.8 - 1.3 x 10⁶) was carried out on ice (0°C) for
150 minutes in a final volume of 70 μl in RPMI medium containing 20 mM HEPES, pH 7.4 and 1 mg/ml bovine serum albumin. Specific binding of [125I]IL-6 to human melanoma cells was calculated after subtracting non-specific binding from the total binding. The non-specific binding was obtained from samples with 250 ng of unlabelled rhIL-6 (Upstate Biotechnology Inc.). Experiments were done by duplicate determinations. Data were expressed by mean and SE of two to three separate experiments.

**Northern Blotting Analysis:**

**Table 2.1: Cell cycle analysis of human melanoma cell lines with or without IL-6 treatment.**

<table>
<thead>
<tr>
<th>IL-6 added (20ng/ml)</th>
<th>%G1</th>
<th>%G2/M</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 added (20ng/ml)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WM 35</td>
<td>52.2</td>
<td>74.7</td>
<td>11.6</td>
</tr>
<tr>
<td>WM 1341B</td>
<td>74.6</td>
<td>83.2</td>
<td>0.6</td>
</tr>
<tr>
<td>WM 902B</td>
<td>67.4</td>
<td>87.3</td>
<td>13.4</td>
</tr>
<tr>
<td>WM 75</td>
<td>63.1</td>
<td>84.4</td>
<td>6.3</td>
</tr>
<tr>
<td>WM 983A</td>
<td>65.2</td>
<td>64.2</td>
<td>1.9</td>
</tr>
<tr>
<td>SKMEL 28</td>
<td>75.3</td>
<td>73.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Poly(A)* RNA was prepared by use of oligo (dT) cellulose (20) from human melanoma cell lines that were cultured in RPMI medium containing 5% FBS. For Northern blot analyses, 3-6 µg of poly(A)* RNA was size-fractionated in formaldehyde-1.0% agarose gels. Hybridizations were conducted at 65°C overnight using [32P]-labelled cDNA probes. cDNA probes for human IL-6 (TaqI/BanII fragment), IL-6 receptor (FspI/EspI fragment), gp130 (AccII/BamHI fragment) and human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH, PsI/XbaI fragment, American type Culture Collection) were radiolabelled with [32P]dCTP and Klenow fragment of DNA polymerase I and random hexamer primers (165,169,423,469).

**B9 Cell Bioassay:**

The IL-6-dependent murine B-cell hybridoma B9 cells were used and cultured as described before (2,354). Conditioned media (CM) of human melanoma cell lines were collected and assayed as follows. Melanoma cells were subcultured in 6-well plates till confluent in RPMI medium containing 5% FBS. 2.5 ml of the same and fresh medium was then added into the culture overnight and CM was collected, called CM1 which contained 5% FBS. Subsequently, cells were washed once with PBS and cultured in 2.5 ml of ExCell 300 medium overnight. The serum free CM2 was then collected. B9 cells were plated and cultured in 96-well plates at 10^5 cells per well with or without CM of melanoma cell culture, rhIL-6 and/or anti-hIL-6 neutralizing antibody at 37°C for two days. Cells were pulse labelled with [3H]thymidine for 4 to 6 hours before harvested in glass fibre filters as described above. Stimulating units of melanoma cell CM were calculated by the concentration of CM that doubled the [3H]thymidine uptake in B9 cells.
Fig. 2.1: IL-6 Induced Time-Dependent Growth inhibition is by G1 Arrest. WM35 melanoma cells were tested for [H\textsuperscript{3}]thymidine incorporation (triangle) after cultured in the absence or presence of hIL-6 (10ng/ml) in 96-well plates for the period of time as indicated. The percentage of cells in G1 phase (closed circle) and S phase (open circle) was determined as described in Materials and Methods. Controls without IL-6 treatment at each time point were considered as 100%
**IL-6 Antisense Oligonucleotide:**

A 15-base antisense oligodeoxynucleotide (TCCTGGGGGTACTGG) was synthesized that is specific for a sequence in the second exon of IL-6 gene was used, as described before by others (240,279,472). The antisense and a control 15 mer sense oligonucleotide were synthesized by pharmacia Biotechnology Service (Toronto, Ontario) and phosphorothioate preparations were made in the Department of Medical Genetics, University of Toronto. To assess the effect of antisense oligonucleotides on the growth of melanoma cell lines, cells were cultured in 24-well plates (10⁴ cells per well) with or without 10μM of the oligonucleotides in RPMI medium containing 5% FBS for 5 days. Cell numbers were counted by trypan blue exclusion in a hemocytometer. Cell number in wells without oligonucleotide was calculated as 100%.

2.4: RESULTS

**IL-6 Arrests Early-Stage Melanoma Cell Growth at the G1 Phase of Cell Cycle:**

Previously, we have found that recombinant human IL-6 inhibited the proliferation of three out of four melanoma cell lines that were derived from early-stage (metastatically incompetent) malignancy (255). These three cell lines are WM35, WM902B and WM1341B, which are grouped and referred to in this paper as 'IL-6 sensitive'. The exception is WM793 cell line that is not growth inhibited by IL-6 but is inhibited by the addition of IL-1 and TNFα. Among the seven human melanoma cell lines derived from either advanced-stage primary tumor or metastases, none was growth inhibited significantly by the addition of IL-6 into culture medium (255). They are WM1361A, WM983A, WM1205, WM9, WM451,
Fig. 2.2: Northern Blot Analysis of IL-6 Receptor and gp130 Gene Expression in Human Melanoma Cell Lines: 3 µg of poly (A)⁺ RNA from each cell line was loaded into each lane in electrophoresis. cDNA probes for IL-6 receptor, gp130 and GAPDH were used as described in Materials and Methods.
MeWo, and SKMEL28, which (including WM793) are referred as to 'IL-6 resistant' group.

Flow cytometric DNA analysis was conducted in cell lines from both IL-6 sensitive and resistant groups. As shown in Fig. 2.1 and Table 2.1, addition of recombinant human IL-6 into culture medium resulted in a time-dependent growth inhibition in WM35 melanoma cell line as assessed by [H³]thymidine incorporation, which corresponded to the decrease in cell

Table 2.2. Scatchard analysis of IL-6 receptor in human melanoma cell lines *.

<table>
<thead>
<tr>
<th></th>
<th>[³H]thymidine uptake</th>
<th>Kd</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(5 ng/ml IL-6)</td>
<td>(x 10⁻¹⁰ M)</td>
<td>(sites/cell)</td>
</tr>
<tr>
<td>CESS</td>
<td>3.55 ± 0.39</td>
<td>2,211 ± 105</td>
<td></td>
</tr>
<tr>
<td>WM 35</td>
<td>30.1 ± 1.4</td>
<td>5.67 ± 1.07</td>
<td>580 ± 53</td>
</tr>
<tr>
<td>WM 1341B</td>
<td>30.7 ± 0.1</td>
<td>1.51 ± 0.10</td>
<td>365 ± 54</td>
</tr>
<tr>
<td>WM 902B</td>
<td>29.2 ± 0.6</td>
<td>2.26 ± 0.14</td>
<td>382 ± 82</td>
</tr>
<tr>
<td>WM 983A</td>
<td>95.8 ± 4.8</td>
<td>1.48 ± 0.23</td>
<td>212 ± 38</td>
</tr>
<tr>
<td>WM 451</td>
<td>91.2 ± 2.7</td>
<td>1.48 ± 0.27</td>
<td>463 ± 9</td>
</tr>
<tr>
<td>SKMEL 28</td>
<td>94.0 ± 5.6</td>
<td>2.86 ± 0.72</td>
<td>415 ± 103</td>
</tr>
<tr>
<td>9-Δ6-1</td>
<td>89.6 ± 5.4</td>
<td>2.34 ± 0.00</td>
<td>755 ± 93</td>
</tr>
</tbody>
</table>

*Data are expressed as mean and SE and CESS cells were used as positive controls.
Fig. 2.3: Scatchard Plot of [125I]IL-6 Binding to Human Melanoma Cell Lines.

Representative data of Scatchard plot analysis from six human melanoma cell lines are shown. Three cell lines in left panel are growth inhibited by IL-6, whereas three in right panel are not growth inhibited by IL-6.
Fig. 2.4: Northern Blot Analysis of IL-6 gene expression in Human Melanoma

Cell Lines: 6µg of poly (A)* RNA preparation from each cell line was used in each lane. Human IL-6 and GAPDH mRNA are both below 18S.
numbers in S phase, and to the increase in cell numbers in G1 phase. The data suggested that the IL-6-induced growth inhibition of early-stage melanoma is mediated by arrest in G1/G0 phase. Changes of cell ratio in G1 and S phase upon addition of IL-6 were not observed in IL-6 resistant advanced-stage melanoma cell lines.

**Evaluation of IL-6 Receptor Status in Melanoma Cell Lines:**

Northern blotting analysis revealed a similar levels of IL-6 receptor gene expression in both IL-6 sensitive and IL-6 resistant cell lines (Fig 2.2). It is known that IL-6 receptor itself does not have signal transduction function and IL-6 action is mediated through gp130 after ligand binding to receptor (165,294). At the RNA level, we did not find marked difference in gp130 gene expression among these human melanoma cell line either (Fig. 2.2).

The biological function of IL-6 receptor was assessed by analyzing $[^{125}\text{I}]$IL-6 binding to the receptor, i.e., Scatchard analysis. As summarized in Table 2.2 and Fig.2. 3, there was no significant difference between IL-6 sensitive and IL-6 resistant groups in terms of $[^{125}\text{I}]$IL-6 binding affinities and binding sites per cell. Cell line 9-Δ6-1 was cloned from WM9 after transfected with an IL-6 antisense expression vector because of endogenous IL-6 production (see below and CL & RSK unpublished observation) and is growth resistant to IL-6 inhibition. In Scatchard analysis, we used CESS cell line as a positive control and obtained a binding affinity and receptor number comparable with those published by Taga et al. (406).

**Endogenous IL-6 Production by IL-6 Resistant Cell Lines:**

Northern blotting analysis of poly (A)$^+$ RNA from these human melanoma cell lines
Fig. 2.5: Stimulating Activities on B9 Cell Proliferation by Conditioned Medium (CM) from Human Melanoma Cell Lines: A) Serum free CM from 11 human melanoma cell lines was assayed in B9 cells. Data are expressed as mean (±SE) of two separate experiments conducted in triplicate determination. B) Addition of anti-hIL-6 neutralizing antibody could abrogate the stimulatory activity of IL-6 and CM2 from melanoma cell lines on B9 cell growth.
revealed that IL-6 mRNA was only detectable in five out of eight cell lines that are resistant to IL-6 growth inhibition. None of the three IL-6 sensitive cell lines had endogenous IL-6 expression (Fig. 2.4). Similar IL-6 mRNA levels were found in another separate experiment. To address the question of whether these five cell lines, WM793, WM1205, WM9, MeWo and WM1361A produce and secret into the medium biologically active IL-6, we tested the conditioned medium (CM) from melanoma cell culture in B9 cell bioassay. CM from three IL-6 sensitive cell lines did not demonstrate significant stimulatory activity in B9 cells as monitored by [3H]thymidine incorporation. While CM from the five IL-6 resistant cell lines with detectable IL-6 mRNA significantly stimulated the proliferation of B9 cells in both FBS containing CM1 (data not shown) and serum free CM2 (Fig. 2.5a). The stimulatory activity on B9 cells by CM could be abrogated by addition of neutralizing anti-hIL-6 antibodies (Fig. 2.5b), indicating that the stimulatory activity is produced by IL-6 secreted from melanoma cells. B9 cell bioassay is consistent with the observation in Northern blotting analysis, and both provide the evidence that these five IL-6 resistant cell lines have endogenous IL-6 production and secret the product into their microenvironment.

Endogenous IL-6 Functions as an Autocrine Growth Stimulator for Melanoma Cells:

Since we have shown that IL-6 is a potential growth inhibitor in human melanoma cell lines that were derived from early-stage (metastatically-incompetent) lesions, the question we asked here was what is the function of endogenous IL-6 in these IL-6 resistant melanoma cell lines. Initial experiments in vitro by adding anti-IL-6 antibody into cell culture medium did not show any changes in [3H]thymidine uptake in melanoma cells with endogenous IL-6 production (Fig. 2.6). Failure of neutralizing Ab to alter the growth in vitro does not exclude
Fig. 2.6: Addition of anti-IL-6 Neutralizing Antibody into Culture Medium Does not Affect the Growth of Melanoma Cell Lines that Produce Endogenous IL-6. Human melanoma cells (5 x 10^5/well) were cultured in 96-well plate for two days with or without IL-6 neutralizing antibody (10 μg/well). Human recombinant IL-6 (12.5 ng/ml) or with IL-6 Ab was added in WM35 and WM902B cell lines to show that IL-6 Ab is biologically active.
the possible intracellular action of endogenous IL-6 in these cells as previously described in other system (240). When a preparation of antisense oligonucleotide to the IL-6 gene was added into cell culture medium, we observed a marked decrease in cell proliferation in IL-6 producing cell lines, but not in non-IL-6 producing cell lines (Fig.2.7), indicating an intracellular action of growth stimulation by IL-6 in the human melanoma cell lines with endogenous IL-6 production.

2.5: DISCUSSION

In recent studies we found a selective growth inhibition of human melanoma cell lines that were derived from patients with early-stage benign (curable) lesions, whereas all seven cell lines obtained from advanced-stage (metastatically-competent) lesions were resistant to IL-6 growth inhibition (255). The results of the present studies do not support the notion that differential [\(^{125}\)I]IL-6 binding to its receptors between IL-6 sensitive and resistant cell lines as being the mechanism mediating the selective inhibition. Northern blotting analysis also revealed similar mRNA levels for both IL-6 receptor and its associated IL-6 signal transducing protein, gp130. Thus, although the mechanism of the resistant phenotype to IL-6 induced growth inhibition in advanced-stage human melanomas remains to be clarified, in all likelihood it does not involve defective or altered cell surface IL-6 receptors.

Endogenous IL-6 production was assessed by both Northern blotting analysis and the B9 cell proliferation bioassay. Surprisingly, five out of eight melanoma cell lines known to be resistant to IL-6-mediated growth inhibition were found to be positive for endogenous IL-6 production. This prompted us to ask whether this endogenous IL-6 had any function on
Fig. 2.7: Effect of IL-6 Antisense Oligonucleotide on the Growth of Human Melanoma Cells. A) WM9 human melanoma cells were plated in 96 well plates and incubated with different concentrations of sense or antisense phosphorothioate oligonucleotides for 2 days. B) antisense oligonucleotide preparation (black solid bars) was added into culture medium (10μM) for 5 days, and then cell number was countered. Data are expressed by mean (±SE) from representative experiment. Cell numbers without addition of oligonucleotide were considered as 100%. Similar data were obtained in another separate experiment.
melanoma cell growth. There are at least three possibilities: 1) IL-6 could still function as a growth inhibitor but in this case the melanoma cells are sufficiently tolerant to overcome the inhibition; a precedent for this is found in TGF-β in which we previously showed that a colon carcinoma cell line was growth stimulated in vivo when transfected with an antisense TGF-β1 expression vector (465); 2) it may be inert functionally as found in three other IL-6 resistant cell lines, which are negative for endogenous IL-6 production; or, 3) it may act as a growth stimulator by an autocrine mechanism as has been found in some hematologic cancers (208,240,278,316,475). To analyze this problem, we added neutralizing antibodies to IL-6 into cell culture medium but this failed to alter the in vitro growth of melanoma cell lines endogenously positive for IL-6 production. However, the consistent growth inhibition we detected by addition of antisense oligonucleotides to the IL-6 gene into cell culture medium clearly indicated that endogenous IL-6 functions as a growth stimulator in these human melanoma cell lines, in all likelihood at an intracellular level. More recently we have found that transfection of an IL-6 antisense expression vector into IL-6 positive WM9 and MeWo melanoma cell lines significantly reduced their growth in nude mice (48) (see Chapter 3). Thus, when taken together with previous observations (255,403) our results strongly suggest that IL-6 acts as a 'bifunctional' cytokine for human melanoma cells during malignant tumor progression: it acts as a growth inhibitor for normal melanocyte (403) and for early-stage primary melanomas by a paracrine mechanism (255) whereas the more advanced-stage form of this malignancy has a distinct tendency to become resistant to IL-6 induced growth inhibition -- and in some cases to use it endogenously as a means of stimulating their own growth. Tumor cell associated IL-6 could also affect tumor growth in vivo by additional
mechanisms, for example by influencing tumor angiogenesis (290) and/or by affecting the ability of the immune system to recognize and destroy (immunogenic) tumor cells (331).

Acquisition of endogenous IL-6 production and its possible contribution in autocrine cell growth have been implicated as a key step in certain types of hematologic malignant tumor progression (193, 208, 240, 316, 475). However, the effects and function of IL-6 in human malignancies of non-haematopoietic origin would appear to be more complicated. For example, endogenous expression of IL-6 has been found in prostate and ovarian carcinoma cells as well as glioblastoma cells with no indication as yet about its function on the growth or other properties of these cancers (382, 433, 451). Growth inhibition or stimulation mediated by IL-6 were reported for human breast carcinoma (73, 311) and renal cell carcinoma, respectively (153, 277). Moreover, in a recent study, no significant effect of human IL-6 on the in vitro growth of more than 26 human malignant non-hematopoietic cell lines, including two cell lines of breast carcinoma was observed (377). Since the latter study involved the use of advanced-stage malignant tumors, exogenous IL-6 might not be expected to have an effect, consistent with our melanoma results. However, many of these tumors may be inhibited by IL-6 in an earlier stage of tumor progression, and conversely, may be stimulated 'cryptically' in more advanced stages by endogenously produced IL-6. Of possible significance in this regard, Sehgal and his associates have reported that the wild-type p53 suppressor gene may normally suppress IL-6 gene expression in epithelial cells (357). Hence, inactivation of the p53 gene by deletion and/or mutation may lead to ectopic production of IL-6 in carcinomas and other types of tumors which undergo such genetic alterations. This may help explain the high frequency of IL-6 production in vitro (see above)
or in vivo (357,405) by solid tumors, since p53 gene mutations are found in as many as 50% or more of all cancers (110). While our results have concentrated on IL-6 as a possible hematopoietic autocrine growth factor in non-hematologic malignancies, it should be noted that other types of hematopoietic growth factors have been implicated as possible autocrine simulators of certain carcinomas. For example, colony stimulating factor-1 (CSF-1) has been implicated to function in such a manner in both human breast and ovarian cancers (199,200,415).

Progressive resistance or 'emancipation' (221) to the action of inhibitory growth factors during tumor progression has been noted in the context of other growth factors, especially TGF-β (59,192,260,285,368,420,470) as reviewed recently by Kerbel (26). Furthermore, in some cases paracrine TGF-β has been shown in some cases to change from acting as an inhibitor to a growth stimulator during transition from low grade to well differentiated high grade advanced malignant cancers (176,192,368,470). However, to our knowledge, there is no precedent for a growth factor functioning as a potential paracrine growth inhibitor during one stage of tumor progression, and as an autocrine growth stimulator at a later, more malignant stage, as shown in this report. Such transitions in responsiveness may contribute to the ability of malignant (metastatically-competent) tumor cells to grow progressively at the primary tumor site, i.e. to manifest a 'growth dominant' phenotype (211), as well as to grow in distant organ sites. Our results have two other important potential implications. First, they imply that IL-6 — and by implication perhaps other members of the hematopoietic growth factor family — may contribute to the growth of solid tumors in a more significant way than previously suspected. Second, they raise the
intriguing possibility of `multi-cytokine resistance' (e.g. to TGF-β and IL-6) and multi-growth factor independence (108) being important aspects of the growth of advanced stage, metastatic, cancers (212).

Acknowledgements

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Chapter 3:

Endogenous Interleukin-6 can Function as an *in vivo* Growth Stimulatory Factor for Advanced Stage Human Melanoma Cells

3.1: ABSTRACT

We have previously shown that a majority of human melanoma cell lines derived from early-stage lesions were growth inhibited by exogenous interleukin-6 (IL-6) in vitro, whereas cell lines from advanced-stage lesions were resistant to such IL-6 induced growth inhibition. Among the resistant melanoma cell lines 50-60% constitutively produced IL-6 which appeared to function as a growth stimulator in vitro, based on the growth suppressive effects of antisense oligonucleotides to the IL-6 gene. The present study was primarily aimed at evaluating whether endogenous IL-6 also functions in vivo as a growth modulator for IL-6 producing and non-producing melanoma cells. To do so we first introduced an IL-6 expression vector into IL-6 non-producing human melanoma cells using WM35, an early-stage (radial growth phase) cell line whose growth is normally inhibited by IL-6, and WM983A, an advanced stage cell line whose growth in vitro is not affected by exogenous IL-6. None of the IL-6 producing transfectants showed a significant alteration in tumor growth in nude mice. Next, two IL-6 producing melanoma cell lines both of which were derived from metastases, MeWo and WM9, and which are growth resistant to exogenously added IL-6, were transfected with an antisense IL-6 expression vector. Several transfectant clones manifested a constitutive decrease in IL-6 gene expression and protein production, and they also gave rise to much smaller tumors with slower growth rates and longer latency periods. However, these IL-6 antisense transfecteds were not growth suppressed in in vitro cell cultures, relative to their respective parental controls. Taken together the results demonstrate that endogenous IL-6 can indeed function as a growth stimulator for human cutaneous melanomas in vivo. This growth stimulatory or survival mechanism remains to be clarified,
but may be paracrine rather than autocrine in nature.

3.2: INTRODUCTION

With respect to its role in the biology of cancer, the pleiotropic 'hematopoietic' cytokine known as interleukin-6 (IL-6) has, until recently, been studied primarily in the context of its importance in hematologic malignancies. However, for a variety of frequently contradictory reasons, there is considerable and growing interest in IL-6 as an important cytokine that is relevant to the biology of solid tumors. These reasons include the following: (i) the tumor cell parenchyma of a broad range of carcinomas and sarcomas has been found to produce IL-6 (380,405,408,433); (ii) a similarly broad range of types of carcinoma cell lines have been reported to produce IL-6 in vitro (60,277,364,382,433,435,451); moreover in a number of cases this endogenous tumor cell IL-6 has been found to function in vitro as an autocrine growth factor, e.g. in renal cell carcinoma (277,408), hepatocarcinoma (22), and cervical carcinoma (109); (iii) normal mesenchymal cells (fibroblasts, endothelial cells) produce large quantities of IL-6 particularly when they are "activated" by adjacent site of tumor growth or inflammation (88,335); (iv) IL-6 can act as a potential stimulator of cellular components of the immune system and this in turn may act to retard tumor growth; indeed there is interest in exploiting IL-6 gene therapy as a immunotherapeutic strategy to treat tumors (18,207,293); (v) release of IL-6 may contribute to cachexia sometimes seen in cancer patients (412); (vi) expression of endogenous IL-6 may modulate the sensitivity of tumor cells to the toxic effects of certain chemotherapeutic drugs and hence act as a potential survival/resistance factor for tumor cells (48); (vii) human recombinant IL-6 is currently under
consideration as an agent to help accelerate recovery of the hematopoietic system of cancer patients after being subjected to high dose chemotherapy or irradiation (437).

Our interest in IL-6 is in connection to its possible function as a modulator of the growth of human cutaneous melanoma, the nature of which can vary depending upon stage of disease progression. Our primary reason for choosing melanoma is the fact that unlike most other types of human cancer, melanoma cell lines are available from every stage of disease progression (160,162). This includes normal melanocytes and precancerous benign atypical nevi, as well as melanomas from early (curable) stages of the disease and others from more advanced (frequently incurable) stages. The former include radial growth phase (RGP) primary lesions as well as early vertical growth phase (VGP) primary tumors, whereas the latter include advanced VGP primary tumors or distant metastases (160,162). The availability of such cell lines facilitates analysis of the role of various growth factors and cytokines, including autocrine factors, in melanoma growth as a function of tumor progression (33,150,160,161,344,361). It is noteworthy that during melanoma progression, a critical step that is a transition from the RGP to the VGP stage of the disease, is associated with loss of physical separation between tumor cells and cytokine (IL-6) producing dermal mesenchyme.

Our laboratory has been primarily concerned with the effects of a family of negative growth regulators and how human melanoma cells progressively acquire resistance to them. In this respect we have found the following: (i) normal melanocytes, dysplastic melanocytes and cells from early stage melanoma cell lines are highly sensitive to a large and structurally diverse family of growth inhibitory cytokines, which includes transforming growth factor-beta
(TGF-β), oncostatin M (OSM), IL-1, tumor necrosis factor-alpha (TNFα), and IL-6 (212,253,255,260); (ii) tumor progression in human melanoma is associated with a progressive acquisition of resistance to all of these aforementioned growth inhibitors, a phenomenon we have termed "multicytokine resistance" (212,223,255,260); (iii) a form of "cytokine switching" can occur whereby a subset (50-60%) of advanced melanomas not only become resistant to the inhibitory effects of exogenous IL-6, but actually begin producing this cytokine (86,251), perhaps even utilizing it as a mitogen (251). Interestingly, in human melanoma cells IL-6 resistance was not associated with any appreciable changes in expression of gp80 or gp130 subunits of the IL-6 receptor. Likewise the function of the receptor complex appeared to be normal in both IL-6 sensitive and resistant cell lines in terms of ligand binding capacity (251) as well as phosphorylation of JAK kinases, activation of APRF transcription factor (R.S. Kerbel, unpublished observations).

The experiments implicating IL-6 as a positive growth regulator for advanced human melanomas were based on in vitro experiments using antisense oligonucleotides to the IL-6 gene (251). Thus, exposure of IL-6 producing melanoma cell lines to such antisense oligonucleotides resulted in a significant growth inhibition of the treated cells. In contrast, no such inhibition was observed when IL-6 negative melanoma cell lines were treated in a similar manner (251). However, it is becoming increasingly apparent that the interpretation of experiments using antisense oligonucleotides can be complicated by a number of potential artefact or methodological difficulties (147,271,395,446). Moreover, the results of such in vitro experiments, even if valid, may not necessarily apply to the in vivo situation. For both these reasons we decided to analyze the effects of constitutively down-regulating IL-6
expression using an IL-6 antisense expression construct, and evaluate the growth properties of the transfectants both \textit{in vitro} and in nude mice. Our results confirm the notion that IL-6 can indeed function \textit{in vivo} as a growth stimulator of advanced stage (malignant) melanoma cells, but may do so by a paracrine rather than an autocrine dependent mechanism. As such the results have a number of potential clinical implications in respect to the use and effects of IL-6 in the context of melanoma biology and treatment. Furthermore to our knowledge this is the first mechanistic study demonstrating that endogenous, tumor cell derived IL-6 can act \textit{in vivo} as a solid tumor growth promoting factor.

3.3: MATERIALS AND METHODS

Construction of antisense IL-6 expression vectors:

The MBAE/H6Δ human IL-6 retroviral expression vector has been described (70).

![Figure 3.1: Antisense IL-6 retroviral expression constructs.](image)

Figure 3.1: Antisense IL-6 retroviral expression constructs. Schematic representation of MBAE/F4 which express antisense human IL-6 mRNA. LTR, long terminal repeats; Neo, neomycin phosphotransferase; β-act, β-actin promoter, B, H, N, and Sc, Bgl II, Hind III, Nhe I and Sac I, recognition sites, respectively.
The MBAE/F4 antisense expression vector for hIL-6 (Fig. 3.1) was constructed by inserting (in a 3' to 5' orientation) a 1.13 kb BamHI-SspI fragment containing the hIL-6 cDNA (169) from pcD-hIL-6 (a gift of DNAX, Palo Alta), after addition of HindIII linkers, into the HindIII site in the retroviral vector MBAE (203) such that antisense hIL-6 transcripts are directed by an internal β-actin promoter/enhancer. Antisense hIL-6 mRNA synthesis was confirmed in MOLT-3 cells using RNase protection assays (Chambers and Hozumi, unpublished observations).

**Retroviral infection of the WM35 early stage human melanoma cell line**

A retroviral expression vector (MBAE/H6Δ) for human IL-6 was used to produce retrovirus in PA317 viral packaging line (70). Cells from the WM35 human melanoma cell line were plated in 60 mm dishes in RPMI medium containing 5% fetal bovine serum (FBS) overnight and subsequently cultured with conditioned medium from the virus infected packaging cell line along with Polybrene (8 μg/ml; Sigma). Variants were selected by the resistance to geneticin (G418) at 800 μg/ml (dry powder weight, GIBCO). Clones were isolated and tested for IL-6 production in both the B9 bioassay and by ELISA of human IL-6 (251).

**Transfection of expression vectors by electroporation**

Since the titre of the retrovirus expressing the IL-6 antisense mRNA was very low, we decided to use DNA transfection techniques for all experiments involving the WM983A, WM9 and MeWo cell lines. Expression vectors of sense and antisense IL-6 gene and their control vectors without the IL-6 gene inserts were transfected into human melanoma cell lines.
Table 3.1 Tumor Growth in Nude Mice of WM35 Human Melanoma Cells and WM35 Variants Obtained by Infection with an IL-6 Retrovirus

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Human IL-6 (pg/ml/10^6 cells)</th>
<th>Tumor Incidence</th>
<th>Tumor size (mm³) Mean (SE)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM35</td>
<td>not detectable</td>
<td>5/5</td>
<td>181.1 (75.6)</td>
<td>58.8 - 516.1</td>
</tr>
<tr>
<td>35-3</td>
<td>400</td>
<td>4/5</td>
<td>314.1 (153.2)</td>
<td>85.7 - 837.8</td>
</tr>
<tr>
<td>35-13</td>
<td>70</td>
<td>1/5</td>
<td>481.6</td>
<td></td>
</tr>
<tr>
<td>35-27</td>
<td>1180</td>
<td>5/5</td>
<td>110.7 (30.8)</td>
<td>21.1 - 208.0</td>
</tr>
<tr>
<td>35-28</td>
<td>2650</td>
<td>0/5</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

35-3, 35-13, 35-27 and 35-28 were cloned after cells from WM35 cell lines were infected with retrovirus expression vector containing the IL-6 gene. Human IL-6 activity was measured in conditioned medium by ELISA. Tumor growth data were obtained at day 71 after subdermal injection (10^6 cells per nude mouse).
by electroporation with Gene Pulser™ (BioRad) at a voltage of 200 to 450 with a capacitance of 500 μF. Cells were then cultured in RPMI medium with 5% FBS for two days before selection through the addition of geneticin (800 μg/ml, powder weight) for the sense and antisense IL-6 infection experiments. Transfected variants were cloned, and initially selected by Southern blotting analysis, Northern blotting analysis, B9 cell assay and ELISA.

Cell culture and assessment of cell growth in vitro

Human melanoma cell lines were cultured as described previously in RPMI 1640 medium containing 5% FBS (251,255), unless otherwise stated. Cell growth was assessed by [³H]-thymidine incorporation assay, as described previously with 5,000 cells per well using 96-well plates in ExCell 300 medium (J.R. Scientific; Woodland, CA) containing 1% FBS (251,253,255). Recombinant human IL-6 was purchased from Upstate Biotechnical Inc. (Lake Placid, NY). Growth rates were also determined by direct cell counting after plating cells in 24-well dishes at 2 x 10³ per well. Cells were trypsinized and their numbers were then assessed using a Coulter Counter (Coulter Electronics Ltd. Bedfordshire, England). A soft agar assay was undertaken by plating in 6-well plates cells in the log growth phase cell growth (5 x 10³) in 0.36% agar over a layer of 0.6% agar, both of which contained RPMI 1640 medium with 5% FCS. The colonies were counted after defined colonies had formed and data were expressed as percentage of cells initially plated. In plating efficiency studies, 250 to 500 cells were cultured in 6-well plates in RPMI medium with 5% FCS for 10 to 14 days. Colonies were fixed in Carnoy's Fixative (2 vol. methanol and 1 vol. glacial acetic acid) and then stained with 0.2% crystal violet. Data were expressed as a percentage of cells initially plated.
Table 3.2 Human IL-6 Production, Growth in Soft Agar and Plating Efficiency of WM983A and its Transfectants

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>IL-6 Conc. (pg/ml/10⁶ cell)</th>
<th>Growth in Soft Agar (%)</th>
<th>Plating Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM983A</td>
<td>N.D. ²</td>
<td>0.99</td>
<td>11.63</td>
</tr>
<tr>
<td>WM983A/MBAE</td>
<td>N.D.</td>
<td>9.19</td>
<td>13.54</td>
</tr>
<tr>
<td>WM983A/ASIL-6</td>
<td>N.D.</td>
<td>7.47</td>
<td>2.14</td>
</tr>
<tr>
<td>WM983A/AS6-10</td>
<td>N.D.</td>
<td>3.39</td>
<td>17.67</td>
</tr>
<tr>
<td>WM983A/IL6-2</td>
<td>91</td>
<td>16.35</td>
<td>19.67</td>
</tr>
<tr>
<td>WM983A/IL6-4</td>
<td>1,990</td>
<td>12.19</td>
<td>79.57</td>
</tr>
<tr>
<td>WM983A/IL6-5</td>
<td>88</td>
<td>13.22</td>
<td>43.30</td>
</tr>
</tbody>
</table>

(1). WM983A/MBAE, WM983A/ASIL-6 and WM983A/AS6-10 were transfectants of WM983A with control plasmid MBAE and antisense IL-6 vector, respectively. WM983A/IL6-2, WM983A/IL6-4 and WM983A/IL6-5 were clones obtained after transfection with the sense IL-6 expression vector. (2). The concentration of human IL-6 was determined by ELISA of conditioned medium. N.D. means not detectable.
Analysis of IL-6 gene expression and protein production by Northern blotting analysis and ELISA

Assessment of human IL-6 gene expression was performed by Northern blotting analysis, as previously described, by use of poly (A) mRNA preparations (251). Human IL-6 cDNA probe (TaqI/BamHI fragment) (169) and human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (PstI/XbaI fragment) (423) were used for the hybridizations. IL-6 protein production in culture medium was analyzed by ELISA. Cells from human melanoma cell lines and their transfected clones were plated in RPMI 1640 medium containing 5% FBS at $10^6$ cells per well in 6-well dishes, and cultured overnight at 37°C. Cells were then washed twice with Excell 300 medium and maintained in 1.0 ml of Excell 300 medium without FBS for 24 hours. Conditioned medium was collected and stored at -20°C, and viable cell numbers were counted in a hemocytometer after trypan blue exclusion. Levels of human IL-6 in conditioned medium was tested using hIL-6 ELISA kit as described by the supplier (Amersham).

Assessment of tumor growth in nude mice

Female athymic nude (nu-nu) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments described. Human melanoma cells cultured in RPMI medium containing 5% FBS were trypsinized and suspended in RPMI 1640 FBS-free medium. The harvested melanoma cell lines in RPMI 1640 medium were injected into mice by subdermal inoculation of 0.5-1 x $10^6$ cells in a volume of 50 µl, as described previously (87). Five to six mice were used for each of the cell lines or clones, per experiment. The same experiment was repeated at least once. Animals were periodically checked and tumor size
Figure 3.2: Tumor growth in nude mice of WM983A cell line and its transfected clones. Melanoma cells (10^6) were injected subdermally into nude mice. Data are expressed as mean (&SE, n=5) from a representative experiment, WM983A (closed cycle), WM983A transfected with control vector MBAE (closed square), or antisense IL-6 vector (closed down triangle), or with sense IL-6 vector of clone 2 (open cycle), clone 4 (open square) and clone 5 (open up triangle).
was measured weekly. Tumor volume (mm$^3$) was estimated from the length (a) and width (b) of the tumor by the formula: $\text{volume} = \frac{ab^2}{2}$ (336).

**Staining of tumor tissue for the presence of blood vessels:**

Tumor tissues were collected, immediately frozen in OCT compound and stored at -70°C until used. Unfixed frozen sections were blocked with 5% BSA, overlayed with GSI lectin from *Griffonia simplicifolia* (Sigma, St. Louis, MO) at the concentration of 2 μg/ml for 30 minutes. Control slides were treated with the lectin inactivated by 0.2 M α-methyl-galacto-pyranoside. The slides were then washed with PBS and treated with rabbit polyclonal antibody against GSI (Serotec, Oxford, England). The color reaction was developed by incubation of the slides sequentially with biotinylated anti-rabbit antibody, peroxidate conjugate and the AEC substrate provided in the Histostain Kit (Zymed, St. Francisco, CA). The sections were counterstained with hematoxylin and mounted with Crystal/Mount (Biomega Corp., CA).

**RESULTS**

**IL-6 gene overexpression alone fails to enhance the tumorigenic properties of early-stage melanoma cells that normally do not produce IL-6**

From our previous work (251,255), we classified human melanoma cell lines into at least three distinct groups in terms of their production and response to IL-6. The first group comprises those cells that are sensitive to IL-6-induced growth inhibition, most of which are derived from early-stage lesions. The second group consists of those cell lines which express a resistant phenotype to exogenous IL-6 induced-growth inhibition *in vitro,*
Figure 3.3: Human IL-6 production in conditioned medium of melanoma cells assessed by ELISA. Conditioned medium was collected in ExCell 300 medium (without serum) 24 hours after culture. Me1, Me4 and Me7 were clones transfected with MBAE/F4 antisense IL-6 expression vector in cells from MeWo cell line, as was W9-10 from WM9.
and which do not produce endogenous IL-6. Finally, the third group includes cell lines whose growth *in vitro* is not affected by exogenously added human IL-6, but which constitutively express IL-6 mRNA and protein; moreover, this endogenous IL-6 appears to function *in vitro* as an autocrine growth factor for these cell lines.

We began our investigation of the role of endogenous IL-6 production in progression of human melanoma by addressing two issues. First, we wished to determine whether the growth inhibitory effect of IL-6 on early stage melanoma cells can be recapitulated under more physiologic conditions, such as upon injection of the cells into nude mice. We decided, therefore, to overexpress IL-6 in the melanoma cells themselves since mouse IL-6 is inactive against human targets. Second, we asked whether such enforced overexpression of IL-6 in IL-6 sensitive cells would be sufficient to confer some degree of resistance to this cytokine in a similar manner as, for example, expression of endogenous TNFα is associated with TNFα resistance in breast and ovarian carcinoma cells (392,464). We chose the WM35 cell line from the first group of melanomas. This cell line was derived from a patient with an RGP lesion (160,205) and was previously shown to be sensitive to growth inhibition *in vitro* induced by a number of exogenous cytokines, including IL-1α, IL-1β, IL-6, TNFα, OSM (253,255) and TGF-β (260). Parental WM35 cells were infected with a retrovirus expression vector encoding the human IL-6 gene. Thirty clones were initially isolated, a majority of which did not expand readily *in vitro*. This may be a reflection of the autocrine IL-6 driven feedback type of growth inhibition (255). For practical reasons, only four clones were chosen for further analysis after G418 selection. All four clones (called 35-3, 35-13, 35-27 and 35-28) secreted detectable amounts of IL-6 protein into the culture medium.
Figure 3.4: Northern blotting analysis of human IL-6 gene expression. 10 µg of poly(A)^+ RNA was loaded in each lane. Probes for human IL-6 gene and GAPDH were used. Endogenous IL-6 message RNA is below 18S as indicated. The hybridization band between 28S and 18S in W9-10 indicates messenger RNA for antisense IL-6.
(Table 3.1). Interestingly, their growth in vitro was not strongly affected by exogenously added recombinant IL-6. However, when these clones were injected into nude mice, there was no significant increase in tumorigenicity compared with the IL-6 negative parental WM35 cell line (Table 3.1). Likewise, a pooled population of infected WM35 showed the same growth rate and tumor take in nude mice compared to wild-type WM35 cells (data not shown).

From the second (IL-6 resistant, IL-6 non-producing) group, a melanoma cell line WM983A derived from a "thick" VGP primary lesion (160,205), was selected for study. cDNA expression vectors transcribing sense and antisense IL-6 mRNA, and the control vector of MBAE (without the IL-6 gene inserts), were used to transfect the WM983A cell line by electroporation. Pooled populations, or clones, were isolated by selection for G418 resistance. Expression of IL-6 gene and production of IL-6 protein were analyzed by Northern blotting and ELISA, respectively. All the IL-6 sense transfectants showed evidence of IL-6 production. As expected IL-6 production was not detected in antisense IL-6 expression or control vector transfections (our unpublished observations, and Table 3.2). The in vitro growth rate of all selected transfectants and of parental WM983A cells were essentially the same, as determined by cell counting when cultured in RPMI medium containing 5% FBS (data not shown). The clone WM983A/IL6-4 had a higher plating efficiency (along with a much greater level of IL-6 production) than other clones (Table 3.2). However, as shown in Figure 3.2, there was no significant difference in tumorigenicity among cell lines transfected with the control vector, or with the sense or antisense IL-6 expression constructs. Another experiment showed the same tumor volume and growth rate between
WM983A and WM983A/MBAE expression vector (data not shown).

**Down-regulation of endogenous IL-6 production results in a significant decrease in tumorigenic competence of advanced stage melanomas**

Two cell lines, MeWo and WM9, that were derived from lymph node metastases of human cutaneous melanomas (162,213), did not respond to exogenously added IL-6 *in vitro* in terms of alterations in cellular proliferation, and were constitutively positive for IL-6 gene expression and protein production (251,255). These are members of the "third" melanoma cell line group in our studies. To explore the potential regulatory effect of endogenous IL-6

**Table 3.3: Transfection of IL-6 expression vector in human melanoma MeWo cells:**

<table>
<thead>
<tr>
<th>hIL-6 (pg/ml/10^6 cells)</th>
<th>Tumor Incidence</th>
<th>Tumor size (mm³, M±SE)</th>
<th>Latency Period (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWo</td>
<td>131</td>
<td>5/5</td>
<td>153.1±16.1</td>
</tr>
<tr>
<td>MeWo/IL-6</td>
<td>821</td>
<td>5/5</td>
<td>406.8±67.5</td>
</tr>
</tbody>
</table>

Production of human IL-6 in culture conditioned medium was determined by ELISA and expressed as pg/ml/10^6 cells/24 hours. Results on tumor growth in nude mice were obtained at day 27 after subdermal injection of 10^6 cells per animal. MeWo/IL-6 was a pooled population after transfected with human IL-6 expression vector from parental MeWo cell line.
on their *in vivo* growth phenotype, stable antisense transfections were conducted to determine whether down-regulation of IL-6 production in these cell lines would alter their growth as solid tumors in nude mice.

The G418-resistant clones were initially screened for antisense construct integration and biological properties by Southern blotting and B9 cell bioassay of conditioned medium (data not shown). Three suitable antisense transfectants called Me1, Me4 and Me7 obtained from the MeWo cell line, and one transfectant clone, W9-10, obtained from WM9 cell line, were selected based on an obvious suppression of endogenous IL-6. As shown in Fig. 3.3, cells from W9-10, Me1, Me4 and Me7 all produced much lower levels of IL-6 protein compared to their respective parental cell lines, as assessed by human IL-6 immunoreactivity of culture conditioned medium. Likewise, Northern blotting analysis of IL-6 gene expression also revealed a decreased level of endogenous IL-6 mRNA in these cell lines (Fig. 3.4). There were no marked changes in IL-6 production in clones transfected with the control MBAE plasmid (data not shown). Transfection of MeWo cells with IL-6 sense expression constructs resulted in a significant increase in IL-6 production (Table 3.3).

To our surprise, in experiments analyzing the characteristics of cell growth *in vitro*, we were unable to detect significant differences in cell growth rate in cell culture between the parental cell line and their respective antisense and sense transfectants, using culture conditions with 1-5% FCS (data not shown). Alteration in IL-6 production through antisense and sense transfection did not have a marked impact on cell growth in soft agar, nor in plating efficiency (data not shown). These results are in apparent contrast to those we previously obtained using antisense oligonucleotides as a means of down-regulating IL-6 production.
(251). Reasons for this apparent discrepancy are summarized in the discussion.

Analysis of *in vivo* tumor-forming ability of MeWo and WM9 and their respective transfectants injected subdermally into nude mice revealed remarkable differences. As shown in Fig. 3.5 and Table 3.4, MeWo cells transfected with the antisense IL-6 expression vector formed much smaller tumors and had slower growth rates compared to parental MeWo cells, or cells transfected with the control MBAE plasmid. In contrast, cells transfected with sense IL-6 expression vector formed larger tumors in nude mice (Table 3.3). Moreover, the *in vivo* volume doubling time of the MeWo antisense transfectants was longer than the parental cells. Similarly, compared to the parental WM9 cells, the IL-6 antisense transfectant (W9-10) subline also formed smaller sized tumors in several separate experiments (Table 3.4). In this case, tumors formed after injection of W9-10 cells had a longer latency period than for WM9 cells, but the growth rate of established W9-10 tumors was the same as that of wild-type WM9 cells. Cells derived from tumors grown in nude mice expressed a G418 resistant phenotype in both Me1 and W9-10 transfectants. In addition, the pooled populations of antisense transfectants from both MeWo and WM9 cells were tested for growth in nude mice, and the results obtained were similar to those with clonal variants (Fig. 3.5 and unpublished observations).

Since the angiogenic properties of tumor cells may have a significant impact on their progressive growth *in vivo* we decided to evaluate this parameter in tumors resulting from injection of WM9 and WM9-10 cell lines or MeWo and Me1 cells. Frozen sections of tumor tissue were stained with the B4 isoelectin from *Griffonia simplicifolia* (BS1) which binds specifically to mouse host stromal cells but not to human tumor parenchyma, and which
<table>
<thead>
<tr>
<th>Cell Lines Transfected with an Antisense IL-6 Expression Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
</tr>
<tr>
<td>Incidence</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td><strong>MeWo and its Transfectants (Day 52)</strong></td>
</tr>
<tr>
<td>MeWo</td>
</tr>
<tr>
<td>Me1</td>
</tr>
<tr>
<td>Me4</td>
</tr>
<tr>
<td>Me7</td>
</tr>
<tr>
<td><strong>WM9 and WM9-10 (Day 53)</strong></td>
</tr>
<tr>
<td>WM9</td>
</tr>
<tr>
<td>W9-10</td>
</tr>
</tbody>
</table>

One million cells in RPMI medium without FBS of a total volume 50 µl were used per animal for subdermal injection. Me1, Me4, and Me7 were cloned from MeWo cells after being transfected with antisense IL-6 expression vector, as was WM9-10 from WM9 cells.
expresses a particular preference for vascular endothelial cells (12). Preliminary FACS analysis of tumor digests stained with this lectin demonstrated that in tumors of both parental and antisense IL-6 transfected MeWo melanoma cells, the stromal component consists of approximately 5-7% of the total cell population (data not shown). The survey of sections of tumor tissue stained with GSI revealed an abundance of blood vessels at the tumor base and tumor-skin interface ranging from 3-20 blood vessels per high power field. The blood vessel density was reduced in the central portion of these tumors. No obvious difference of blood vessel density was detected in tumors of similar size generated by parental or antisense IL-6 transfected cell lines (data not shown). This suggests that changes in IL-6 secretion do not bring about any appreciable reduction of angiogenesis. This is consistent with the fact that a direct angiogenic activity of IL-6 has not been demonstrated thus far, although up-regulation of the IL-6 gene expression has been observed in endothelial cells responding to angiogenic physiological stimuli in murine reproductive organs (290).

3.5: DISCUSSION

The experiments in this study were aimed at helping to clarify the possible functional significance of endogenous tumor cell IL-6 production by solid tumors, particularly human cutaneous melanoma. Considering the extremely broad spectrum of solid tumors known to produce IL-6, either in vivo and in vitro (see 3.2 Introduction), and the diverse pathologic and biologic effects of IL-6, this is an issue of considerable clinical significance. It is well known that IL-6 can function as an autocrine growth factor in various hematologic malignancies such as myelomas and lymphomas (4,208,240,475). In addition, our previous
studies had suggested that, similar to renal cell carcinoma (277), hepatocarcinomas (22) and cervical carcinomas (109), IL-6 can function in vitro as an autocrine growth stimulating factor for a significant subset (approximately 50%) of advanced stage cutaneous melanomas (251). This was all the more interesting in view of the fact that cell lines from early stage melanomas do not express IL-6, and indeed, are strongly growth inhibited by exposure to exogenous IL-6 (223,255). These results suggest that the effects of IL-6 on melanoma cell growth can switch in a rather dramatic fashion as a function of disease progression. The nature of this switch in cytokine responsiveness has the potential to augment the growth advantage and aggressiveness of metastatically-competent melanoma cells (212,255).

The results reported in this study would appear to confirm the notion that endogenous IL-6 can function as a growth stimulant for advanced stage melanoma cells which normally produce it. However, the evidence implicates a host-dependent rather than autocrine process as being involved. Thus, while a significant suppression of melanoma tumorigenicity in nude mice was indeed observed in variants constitutively suppressed for IL-6 expression by transfection of an antisense IL-6 expression construct, these same variants did not show any detectable growth disadvantage in tissue culture.

It is noteworthy that our previous experiments which implicated IL-6 as an autocrine growth factor depended exclusively on the use and effects of antisense oligonucleotides to a portion of the second exon of the human IL-6 gene (251). However, there are increasing numbers of reports in the literature documenting instances where the apparent sequence-specific effects of antisense oligonucleotides on gene expression can in fact be due to a variety
Figure 3.5: Tumor growth of human melanoma cell line MeWo and its transfectants in nude mice. Melanoma cells (5 x 10^5) were injected subdermally in nude mice and the tumor growth was checked periodically. Data are expressed as mean and SE (n=5) of a representative experiment using MeWo (closed cycle), MeWo transfected with control MBAE plasmid (closed square), and MeWo transfected with antisense IL-6 plasmid of clone 1 (Me1) (open cycle), clone 4 (Me4, open up triangle) and pooled population (open down triangle).
of ill-defined non-specific mechanisms (147,395,446). In this regard phosphorothioate oligonucleotides can sometimes bind to certain proteins, especially if the former contain four continuous guanosine residues, (i.e. the "G-quartet") (395). In our IL-6 antisense oligo experiments we used a 15 base antisense oligomer of the sequence TCCTGGGGGTACTGG (251). Hence, it is conceivable that the presence of five continuous guanosines might have caused some non-specific effects. If so, it is curious that the growth suppression we previously observed occurred only in melanoma cell lines known to express IL-6 and not in cell lines negative for endogenous IL-6 production (251). The nature of this possible non sequence-specific effect and whether it involves the G quartet is under investigation in our laboratory.

Our present results are nevertheless consistent with the hypothesis that IL-6 ultimately functions as a growth stimulant for malignant human melanoma cells in vivo (251). In fact, to our knowledge, this is the first mechanistic study showing such an effect of IL-6 in vivo. How it comes about remains to be clarified, but clearly some kind of paracrine mechanism involving host cells and/or tissues would appear to be implicated. For example, tumor cell IL-6 may directly or indirectly trigger the production in adjacent host endothelial cells, fibroblasts or other types of stromal or inflammatory cells, of one or more growth factors which may then stimulate the growth of melanoma cells in a "two-way" paracrine fashion. Such growth factors could include, to cite one example, hepatocyte growth factor/scatter factor (HGF/SF), which is produced by connective tissue cells, is regulated transcriptionally by IL-6 response elements (247), and is a known paracrine stimulating growth factor for melanoma cells (151). Other possible candidate host paracrine growth factors include insulin-
like growth factor 1 (IGF-1) and epidermal growth factor (160-162,205). In addition, such growth factors may also act as "survival" factors to protect melanoma cells from spontaneous or environmentally-induced programmed cell death. Indeed, the possibility remains that endogenous IL-6 itself could act as a survival factor for melanoma cells. In this regard, it is well known that IL-6 can function as a survival (anti-apoptotic) growth factor for a variety of hematopoietic/lymphoid cells (242,245). In leukaemic cells IL-6 can abort apoptosis induced by the wild type p53 suppressor gene (478) up-regulate expression of bcl-X, (369,478) and possibly other survival genes as well.

Finally, an analysis of our results would be incomplete without some comments regarding recently published results demonstrating that IL-6 produced by tumor cells, especially after IL-6 gene transfection, can indirectly and strongly suppress the growth of solid tumors in normal syngeneic mice through host mediated immunologic mechanisms (18,207,293). For example, several groups have reported that transfection and expression of the IL-6 gene in various mouse tumor cell lines results in a relative suppression of tumorigenicity of the transfected cells in normal syngeneic mice, but not necessarily in immune-suppressed hosts. However, we would like to draw attention to the fact that the aforementioned studies on IL-6 transfected mouse tumor cells (207,293) all employed immunogenic mouse tumors such as B16 melanoma or Lewis lung carcinoma (163,164). In such cases, boosting of the immune system by tumor cell secreted IL-6 would indeed exert a powerful anti-tumor effect through the immune system, even if IL-6 can function as a potential growth stimulator. It is highly questionable whether human tumors including melanomas, express the degree of immunogenicity often associated with long-term
transplantable mouse tumors. We would also point out that, as summarized above, many human cancers have been found to produce IL-6 \textit{in vivo}. This would seem inconsistent with the idea that the expression of IL-6 by tumor cells can act as a potent stimulus to suppress tumor growth since this phenotype should presumably be selected \textit{against}, not \textit{for}, during tumor progression.

These considerations merely serve to emphasize the view that the effects of endogenous and exogenous IL-6 on tumor growth can be quite pleiotropic and complex. The final outcome may be a balance involving many opposing effects, including increased or suppressed local tumor angiogenesis (290), increased immunogenicity of the tumor cells, direct autocrine effects on cell growth, and the potential of IL-6 to promote a systemic state of cachexia (412). All of these will have to be ultimately considered in the context of using cytokine encoding genes - including IL-6 - for gene therapy of cancer, along with the fact that many types of solid human cancers \textit{already} produce IL-6. Our results simply highlight the fact that "hematopoietic" cytokines such as IL-6 may have unanticipated growth enhancing effects of solid tumor cells - especially those associated with highly advanced and malignant stages of tumor growth, and thus may also have undesirable consequences for the use of IL-6 as a myelorestorative agent after chemotherapy or irradiation.

**ACKNOWLEDGMENTS**

I sincerely thank Dr. Meenhard Herlyn for his generous support of my study by providing the WM series of human melanoma cell lines. I also like to express my thanks to Drs. C.A. Chambers and N. Hozumi of Mount Sinai Hospital Research Institute for their
permission to use their antisense and sense IL-6 gene expression vectors, and to Mrs. C. Sheehan for her excellent technical help for this study, for which the three were co-authored in the publication.
Chapter 4:

Increased Resistance to Oncostatin M-Induced Growth Inhibition in Human Melanoma Cell Lines That were Derived from Advanced-Stage Lesions.

4.1: Abstract

Human melanomas can become progressively resistant to the growth inhibitory effects of a broad family of structurally diverse cytokines which includes interleukin-6 (IL-6). Uncovering this "multicytokine resistance" was made possible by the availability of cell lines established from early-stage radial growth phase or vertical growth phase primary melanomas as well as more advanced primary lesions and distant metastases. Because Oncostatin M (OSM) is also a member of the IL-6 family we evaluated the effects of this cytokine on the growth of human melanoma cell lines obtained from different stages of disease progression. The results showed that three different cell lines derived from early-stage melanomas were strongly growth inhibited by OSM, similar to IL-6. Three cell lines, established from advanced-stage melanomas, were growth inhibited by OSM, but a much higher range of (ten fold) concentrations were required to obtain 50% growth inhibition; these cell lines were not inhibited by IL-6. Three other cell lines that were "IL-6 resistant" (two of which were advanced stage) were also found to be OSM resistant. Only one advanced stage IL-6 resistant cell line was found to be highly sensitive to OSM-mediated growth inhibition. In addition, we found that variants isolated from early-stage WM35 melanoma cells that possess a much more aggressive tumorigenic phenotype in nude mice were significantly more resistant to both OSM and IL-6 mediated growth inhibition. The results demonstrate that OSM can function as a growth inhibitor of human melanoma cells, but that its ability to do so is progressively diminished or lost with disease progression. This finding is consistent with the concept of acquired "multicytokine resistance" during melanoma progression. In addition, the differential effect of IL-6 and OSM on some of the human melanoma cell lines implies the
existence of a "private" OSM signalling pathway in addition to a "public" one.

4.2: Introduction

The growth advantage of cancer cells over their normal cellular counterparts is thought to be due in part to acquisition of relative resistance to certain autocrine or paracrine growth inhibitory cytokines (113). Foremost among these are members of the transforming growth factor-beta (TGF-β) family, such as TGF-β1 and TGF-β2 (113). Whereas normal epithelial cells from a variety of organs are strongly inhibited by exposure to low concentrations of TGF-β, corresponding carcinomas derived from these epithelial cells are frequently resistant to this inhibition (113). The same is generally true for leukemias and lymphomas (113).

More recent studies have shown that resistance to TGF-β is frequently progressive, i.e. the more biologically advanced and aggressive a lesion, the greater the degree of resistance its cells have to TGF-β-mediated growth inhibition (212). This has been shown in human cancers such as skin tumors (122), glioblastomas (192), and colorectal carcinomas (176,265,470). We have also observed progressive TGF-β resistance in human malignant melanomas (260). Thus cell lines established from early-stage radial growth phase (RGP) or "thin" vertical growth phase (VGP) primary melanomas are growth inhibited by exposure to TGF-β1 (as are normal melanocytes) whereas most cell lines established from more advanced VGP primary melanomas or melanoma metastases are resistant, or partially resistant to this inhibition (260). A similar pattern has been observed when studying more aggressive variants isolated from early-stage human melanomas (223).
The relative resistance of advanced stage metastatically-competent melanomas to TGF-β is also accompanied by a similar relative resistance to other, structurally unrelated cytokines. These include interleukin-6 (IL-6), interleukin-1α (IL-1α) and tumor necrosis factor alpha (TNFα), as recently described by us (251,255). We have coined the term "multicytokine resistance" to describe this phenomenon (212): it may be a major factor in endowing melanoma cells with an ability to grow in the foreign environment of the dermal mesenchyme and achieving "clonal dominance" of metastatically-competent cells within primary tumors (212,214).

The multicytokine resistance phenomenon, and the finding that it includes IL-6, prompted us to determine whether another "hemopoietic" cytokine -- oncostatin M (OSM) -- can behave as an inhibitor for early stage human melanomas and whether its ability to do so is lost in advanced stage lesions. OSM is a glycoprotein that was originally isolated from the conditioned medium of a human histiocytic leukemia cell line (U937) by its ability to inhibit DNA synthesis of A375 human melanoma cells (57,484). The cDNA sequence of OSM, which was cloned shortly thereafter, revealed that OSM is one of the members of the interleukin (IL) 6 family of cytokines (263,350). There are several members in this family, including IL-6, OSM, leukemia-inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), ciliary neurotrophic factor, and myelomonocytic growth factor (29,263). There is a closer relationship among IL-6, OSM and LIF in terms of the ligand-binding and signal transducing activities (126,127,246). It also appears that OSM can mimic most of the biological activities of IL-6 (59,278,297).

The main purpose of this report was to test the effects of OSM on the growth of a
large panel of human melanoma cell lines established from various stages of disease progression. Evidence was obtained to show that its capacity to act as an inhibitor does indeed diminish with tumor progression. However, there was a discrepancy in the degree of growth inhibition induced by IL-6 and OSM in some of the lines, implying the existence of a distinct signalling pathway for the action of OSM in melanoma cells, in addition to the pathway in common with IL-6.

4.3: MATERIALS AND METHODS

Cell lines and other reagents:

Human melanoma cell lines of WM series were obtained kindly from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). The origins and establishment of these cell lines were described before in Chapter 1. A375 cell line was established back in 1973 with no clue regarding the pathological classification (130). The variants of WM 35 cell line (P1P, P2P and P3P) were generated after in vivo passage and selection in nude mice. These cells derived from tumors in nude mice were proven to be human origin without contamination of mouse cells (223,260). P1P, P2P and P3P were referred to as the pooled populations of in vivo passage 1, 2, and 3, respectively as described before by us (223).

Recombinant human IL-6 was purchased from Upstate Biotechnical Inc. (Lake Placid, NY). Recombinant human OSM was purchased from Pepro Tech Inc. (Rocky Hill, NJ) with a ED50 of 2ng/ml in TF-1 cells. Recombinant human LIF was purchased from R & D Systems (Minneapolis) with biological activity (ED50) of 0.15 to 0.3 ng/ml in TF-1 cells. ExCell 300 medium was from J.R. Scientific; Woodland, CA.
Cell culture and $[^3]$Hthymidine uptake assay:

Cell culture and $[^3]$Hthymidine uptake assay was conducted essentially as described previously (88,255). Human melanoma cells were maintained in RPMI medium containing 5% FBS. The growth assay was done in ExCell 300 medium containing 1% FBS and various concentrations of cytokines in 96-well plates in a final volume of 150μl. After 2 days of incubation with cytokine, cells were pulse labeled with $[^3]$Hthymidine for 4 to 6 hours before being harvested into Printed Filtermat A in a Titertek Cell Harvester 530. The residual radioactivity on the filter was counted in a 1205 Betaplate™ scintillation counter (Wallac, Gaithersburg, MD). The radioactivity in tested wells was calculated against control wells without cytokine treatment that was considered as 100%. ID50 was calculated as the concentration of cytokine that caused 50% inhibition of $[^3]$Hthymidine uptake and expressed by the mean value from two to six separate experiments that were performed by triplicate determinations.

4.4: RESULTS

An increased resistance to OSM-induced growth inhibition in human melanomas of advanced-stage: The effect of OSM on human melanoma cell lines derived from different clinical stages was tested in ten cell lines as listed in Table 4.1. In comparison with the response to OSM, IL-6 effect on these cells was cited from our previous work (255). As summarized in Table 4.1, three out of four cell lines that were derived from early-stage melanoma were growth inhibited by addition of recombinant OSM, which is the same as the response to IL-6. However, there is a difference in sensitivities to the growth inhibition
induced by IL-6 or OSM. WM 902B is 4.5 times less sensitive to OSM, but 10 times more sensitive to IL-6, than WM 1341B, both of which were derived from thin VGP lesions (255). The exception in this category of early-stage melanoma is the WM793 cell line that was not growth inhibited by OSM, which is in accordance with resistance to IL-6 (255).

Among cell lines that were derived from advanced-stage melanomas, two did not response to exogenously added OSM (WM 983A, and WM 1361A), which is in agreement with that to IL-6. However, three cell lines (WM 9, WM 451 and SKMEL 28) displayed a certain degree of response, but it was much weaker than WM 35, WM 1341B and WM902b, to OSM-induced growth inhibition although they are not growth inhibited by addition of IL-6.

**Differential effect of OSM on human melanoma cell lines with IL-6 and LIF:** The discrepant responses to IL-6 and to OSM of human melanoma was found in four of the six cell lines from advanced-stage lesions (Table 4.1). One example is the MeWo cell line. MeWo cells were growth inhibited by OSM at relatively low concentration but not by addition of exogenous IL-6 (Fig. 4.1a). In contrast, our work has shown that IL-6 acts as an autocrine growth stimulator for MeWo cells (251).

Recent studies have shown that OSM may share receptor with LIF (126) and both use gp130 as their receptor and/or signal transducer (127,246). However, when the effect of LIF on human melanomas was tested, there was no significant growth inhibition or stimulation on our melanoma cell lines, such as WM 35, WM 1341B, WM 902B, WM 9, WM 451 (Fig. 4.1b and our unpublished observations). Besides LIF, the possible involvement of G-CSF in the growth regulation of human melanomas was also tested, but we were unable to observe any effect of G-CSF on DNA synthesis in vitro in our melanoma cell lines (our unpublished
Table 4.1: Summary of the effect of OSM on \[^{3}H\]thymidine uptake in human melanoma cell lines that were derived from different malignant stages.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Response</th>
<th>ID50 (ng/ml)</th>
<th>ID50 (ng/ml) (to IL-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>to OSM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early-stage melanoma cell lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WM 35</td>
<td>Early-stage RGP (primary)</td>
<td>I</td>
<td>16.4</td>
<td>1.5</td>
</tr>
<tr>
<td>WM 902B</td>
<td>Early-stage VGP (primary)</td>
<td>I</td>
<td>32.3</td>
<td>1.0</td>
</tr>
<tr>
<td>WM 1341B</td>
<td>Early-stage VGP (primary)</td>
<td>I</td>
<td>7.2</td>
<td>14.0</td>
</tr>
<tr>
<td>WM 793</td>
<td>Early-stage VGP (primary)</td>
<td>n.r.</td>
<td>--</td>
<td>n.r.</td>
</tr>
<tr>
<td>Advanced-stage melanoma cell lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WM 983A</td>
<td>Advanced-stage VGP (primary)</td>
<td>n.r.</td>
<td>--</td>
<td>n.r.</td>
</tr>
<tr>
<td>WM 1361A</td>
<td>Advanced-stage VGP (primary)</td>
<td>n.r.</td>
<td>--</td>
<td>n.r.</td>
</tr>
<tr>
<td>WM 9</td>
<td>Metastasis</td>
<td>I</td>
<td>376.4</td>
<td>n.r.</td>
</tr>
<tr>
<td>WM 451</td>
<td>Metastasis</td>
<td>I</td>
<td>459.0</td>
<td>n.r.</td>
</tr>
<tr>
<td>MeWo</td>
<td>Metastasis</td>
<td>I</td>
<td>48.9</td>
<td>n.r.</td>
</tr>
<tr>
<td>SKMEL 28</td>
<td>Metastasis</td>
<td>I</td>
<td>223.6</td>
<td>n.r.</td>
</tr>
</tbody>
</table>
Legends to Table 4.1:

1. [3H]thymidine uptake was tested in 96-well plates in the absence or presence of human recombinant oncostatin M (OSM). "I" represents a growth inhibition of cells OSM in the concentration ranges from 0.1 to 100 ng/ml, compared to controls (considered as 100%). ID50 was calculated by the dose-dependent inhibition at 50% in DNA synthesis and expressed as ng/ml of OSM. ID50 for WM 9, WM 451 and SKMEL 28 was obtained by testing the responses to OSM up to 500 ng/ml. "n.r." means no response (no stimulation, no inhibition). Data were obtained from two to six separate experiments performed by triplicate determinations.

2. Growth response to exogenously added IL-6 in culture was cited from Table 1.2 of Chapter 1 observations).

**Additive effect of IL-6 and OSM on the growth inhibition in human melanoma cells:** The combined effect of IL-6 and OSM was investigated by use of A375 cell line. As shown in Fig. 4.2, there is clear additive effect of growth inhibition on A375 cells between IL-6 and OSM. A similar additive effect of IL-6 and OSM was also observed in another human melanoma cell line, WM 35 that was derived from patient with radial growth phase lesion (data not shown). The growth of WM 35 cells was inhibited by IL-1, IL-6, TNFα (255), as well as TGFβ (260).

**Increased resistance to OSM-induced growth inhibition in experimentally developed tumor progression system:** WM 35 is one of the few cell lines that was derived
Fig. 4.1: Differential response of $[^3]H$thymidine uptake to administration of IL-6, LIF and OSM. $[^3]H$thymidine uptake rate was tested to estimate cell growth in response to IL-6, LIF and OSM. Data are expressed as mean and SE from one representative experiment performed by triplicate determination. Cells without cytokine treatment were considered as controls (100%). A: Response of MeWo cells to IL-6 (open triangle) and to OSM (closed circle). B: Response of WM 1341B cells to LIF (open circle) and to OSM (closed circle).
from human melanoma of radial growth phase (160). In order to establish an experimental system to mimic clinical development of human melanoma progression, cells from WM 35 were injected into nude mice with Matrigel, which could promote the tumor growth. The subline of WM 35 was then developed from tumors in nude mice. Cells adapted into culture have been shown to be human origin and to have an increased resistance to the growth inhibition induced by IL-6, IL-1, TNFα and TGFβ (223,260). By use of these variants of WM 35, we found here that there was a dramatic increase in the resistant to OSM-induced growth inhibition (Fig. 4.3).

4.5: DISCUSSIONS

Oncostatin M was originally purified and characterized as a cytokine with growth inhibitory properties for the human melanoma cell line called A375 (57,484). The results of our studies confirm the conclusion that OSM can inhibit the growth in vitro of human melanoma cells, but with one important qualification: that this property is frequently attenuated, or lost altogether, as melanomas progress. Thus, three of four independent early-stage melanoma cell lines were found to be strongly growth inhibited by exogenous OSM, whereas only one of seven advanced stage melanoma cell lines was comparably inhibited. Of the other six cell lines, three were OSM resistant (in terms of growth inhibition) and three were inhibitable; however much higher concentrations of OSM were required to achieve 50% inhibition of cell growth in these three cell lines (as measured by thymidine incorporation) in comparison to the three inhibitable early-stage cell lines. Moreover, when genetically related variants of early-stage poorly tumorigenic WM 35 cells, selected for high grade-
Fig. 4.2: Additive effect of IL-6 and OSM on growth inhibition in human melanoma cells. \(^3\)H]thymidine uptake rate was measured in the presence or absence of IL-6 or OSM in A375 human melanoma cell line. Left: increased amount of OSM without (closed circle) and with 1.0 ng/ml IL-6 (open circle). Right: increased amount of IL-6 without (closed square) and with 9.33 ng/ml OSM (open square).
tumorigenicity in nude mice (223), were examined, they manifested a significant degree of resistance to OSM mediated growth inhibition. Taken together, the results show that OSM can be added to the list of cytokines known to be strong inhibitors of most cell lines established from early-stage melanomas but whose capacity to bring about such growth inhibition is diminished or lost with melanoma progression (223,255,260). This list includes TGF-β1, IL-6, IL-1α, IL-1β and TNFα (see Table 1.2 and references (255,260)). The significance of OSM to this "multicytokine resistance" (212) remains to be determined as well as the phenomenon itself in terms of its contribution to tumor growth and metastatic spread, in vivo.

The possible mechanisms to account for the differential response to OSM and IL-6 in the melanoma cell lines we analyzed are unclear. The genes for receptors for IL-6 have been cloned and are known to be composed of two components, gp80 with high binding activity for IL-6, and gp130, which has no IL-6 binding activity but which mediates IL-6 signal transduction upon IL-6 binding to gp80 (218). The structure of OSM receptor complexes is poorly understood. It is known that OSM can bind to both gp130 and the LIF receptor (126,127,218,246). Our previous work indicated that differential response to IL-6 was not due to differences in gene expression of gp80 and gp130, nor to IL-6 ligand binding activity (251). Whether this is the case for OSM has yet to be determined in terms of ligand binding activity.

The non-responsiveness of our melanoma cells to LIF supports a previous report showing differential activity of LIF comparing to OSM and IL-6 on A375 cells even though IL-6, LIF and OSM are all effective in the terminal growth arrest and differentiation control
Fig. 4.3: [³H]thymidine uptake in response to OSM and IL-6 in WM 35 cell line and its in vivo selected variants. Cell growth was tested by [³H]thymidine uptake rate in the presence or absence of OSM (A) and IL-6 (B). Data are expressed as mean and SE of triplicate determination from representative experiment. Cell lines used were parental WM35 (closed cycle), and the pooled population, the P1P (open cycle), P2P (closed up triangle), and P3P (open down triangle), after one, two, and three times passage in in vivo selection in nude mice (see materials and methods).
in myeloblastic M1 murine leukemia cells (59). Since there is a strong discrepancy in the response to IL-6, OSM and to LIF in some of the advanced-stage melanoma cell lines, we would suggest that there are distinct signal mechanisms among IL-6, OSM and LIF in addition to their common pathway, i.e. there may be both "private" and "public" (gp130) pathways of action, at least within the melanocytic cell lineage.

In summary, our results show yet another member of the hemopoietic family of cytokines – Oncostatin M – can function as a strong paracrine growth inhibitory for human melanoma cells, provided the cells are derived from early stage primary tumors. The capacity to inhibit is weakened in most of the advanced stage lesions that were examined. OSM is known to be made by macrophages and T lymphocytes -- cells which can be found in the dermis and associated with growing primary melanomas. Hence, there is the distinct possibility that OSM may help contribute to the growth advantage of small numbers of metastatically-competent melanoma cell variants residing within early-stage primary melanomas in vivo. This may assist such variants to overgrow their less malignant counterparts and help to achieve "clonal dominance" in more advanced primary melanomas (211,212).

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As outlined in the final section of the Introduction chapter, it was shown previously in this laboratory that normal dermal fibroblastic cells can inhibit the proliferation of early-stage RGP and 'thin' VGP derived human melanoma cell lines whereas such inhibitory effects are lost on melanoma cells obtained from more advanced-stage (metastatically-competent) lesions (88). The findings summarized in this thesis and in published papers revealed that the molecule responsible for this differential growth inhibitory activity is IL-6, a pleotropic "haematopoietic" cytokine (Chapter 1). It is shown that the loss of responsiveness to IL-6-induced growth inhibition in advanced-stage melanomas is not due to an apparent lack of receptors, based on studies of both Northern blotting and ligand binding Scatchard analysis (Chapter 2). Studies of IL-6 gene expression by Northern analysis and protein production by B9 cell bioassay and ELISA of melanoma cell culture medium indicate that about half of IL-6 resistant cell lines (but not the sensitive cell lines), produce biologically active IL-6 (Chapter 2). Attempts to down-regulate endogenous IL-6 production in melanoma cells by use of an antisense strategy indicates that endogenously produced IL-6 in some advanced-stage human melanoma cells can function in vivo as a growth stimulatory factor for tumors, as assessed in a nude mice growth assay (Chapter 3). In addition to IL-6, a similar development of resistance was also found in advanced-stage melanomas with respect to the growth inhibition induced by several other cytokines, such as IL-1α, IL-1β, tumor necrosis factor α, and oncostatin M (Chapters 1 and 4). This led to the formulation of the concept of "multicytokine resistance" in cancer - or at least melanomas. In summary, it was shown that there is a transitional change in response to IL-6-regulated cellular proliferation in human melanoma cells during disease progression, from growth inhibition, to resistance, and finally
to growth stimulation. Considering the abundance of cytokines in the microenvironment around melanoma cells in vivo, such a transition may help melanoma cells to overcome the inhibitory forces exerted by IL-6 and other cytokines, and hence progress to become metastatically competent, and eventually overgrow their non-metastatic counterparts at the primary site, and generate distant metastases.

5.1 Multi-growth factor independence and multi-growth inhibitor resistance of advanced tumors

i): Increased resistance to paracrine growth inhibitors expressed by advanced-stage melanomas:

Attempts to identify a fibroblast-derived (paracrine) growth inhibitor of human melanoma cell lines obtained from early stage diseases resulted in the discovery of the phenomenon that some haematopoietic cytokines, such as IL-6, may play a dual role in the growth of human solid tumors. That is IL-6 may switch from paracrine growth inhibitor to a growth stimulator depending upon the stage of tumor progression. (212, 223, 251-255). In addition to IL-6, we found that other cytokines, such as TNFα, IL-1α, IL-1β, and OSM also contribute to multicytokine resistance. These cytokine had been considered for the most part to be important modulators of immunity, acute phase protein responses and growth of haematopoietic malignancies (23, 100, 209, 434, 439).

In a parallel and simultaneous study in this laboratory, a progressive increase in resistance to TGFβ-induced growth inhibition as a function of melanoma progression was observed by John MacDougall, a Ph.D. student (260). Thus, TGFβ inhibited the growth of
normal melanocytes, dysplastic nevus cells as well as all four early stage melanoma cell lines that were tested. Such growth inhibitory responses were absent or reduced in more than half of melanoma cell lines that were derived from metastatically-competent lesions obtained from either primary or metastatic tumors (260).

It was also reported at the time that the present work was being undertaken that addition of IL-1, TNFα, IL-6 and TGFβ all caused a growth inhibition of normal melanocytes (260,403). From the present findings, it is clear that the growth inhibition induced by these structurally unrelated growth factors is retained to varying degrees by melanoma cell lines obtained from early stages of disease progression, but is lost or diminished in cell lines obtained from advanced-stage lesions. Among the four melanoma cell lines available to us that were derived from tumors of early-stage lesions, three of them (WM35, WM 1341B, and WM 902B) were growth inhibited by IL-6 and OSM, whereas two of them (WM 35 and WM 793) were inhibited by IL-1α, IL-1β and TNFα (see chapters 1 and 4), and all were found sensitive (albeit to different degrees), to TGFβ (260). Taken together, any one of the four early stages human melanoma cell lines is sensitive to at least three of the five growth inhibitors tested (IL-1, IL-6, TNFα, TGFβ and OSM). The only early stage melanoma cell line that was found, like normal melanocytes, to be sensitive to all five cytokines, was WM35, which was derived from a RGP melanoma.

In sharp contrast, such negative effects on growth are greatly diminished, or totally absent, in melanoma cell lines that were derived from tumors at advanced-stages of disease progression, whether from primary tumors, "thick" VGP or from secondary (metastatic) tumors. Such increased resistance to growth inhibition does not necessarily occur in every cell
line in response to all inhibitors. Some advanced-stage melanoma cell lines were resistant to the growth inhibition mediated by some but not by other inhibitors. For example, the MeWo cell line in vitro was not growth inhibited by IL-1α, IL-1β, IL-6, TNFα, or TGFβ, but, interestingly, was growth inhibited by OSM. WM 9 was growth inhibited only by TGFβ whereas it remained resistant to all other four growth inhibitors. Some advanced-stage melanoma cell lines such as WM451, SKMEL 28 and WM1361A, were found to be resistant in vitro to the growth inhibition induced by all five growth modulators tested. Therefore, these results provided strong evidence showing that advanced-stage melanoma cell lines are phenotypically different from those of early-stage in terms of their response to negative growth regulators in that advanced-stage tumor cells, whether from primary or secondary sites, tend to become resistant not only to one but to a number of such potential growth inhibitors (212,252). Hence the term "multicytokine resistance" was coined to describe this phenomenon.

These multicytokine resistant phenotypic changes in advanced melanoma cells have been confirmed and supported by results recently obtained using different experimental strategies. Melanoma cell lines of different stages were derived from tumors of different patients, i.e. they are not genetically related. To recapitulate the malignant progression experimentally, some attempts have been made in this laboratory by others to generate variant sublines expressing a more aggressive tumorigenic phenotype from such early stage cell lines as WM35 and WM 1341b. One of the strategies used was to mix non-tumorigenic melanoma cells with Matrigel and inject the mixture into nude mice to select for higher tumorigenic variants. After two cycles of selection in nude mice, the variant sublines behaved like more
advanced stage melanoma in vivo and manifested an increased resistance to the growth inhibition induced IL-1, IL-6 and TGFβ in vitro (223). The other approach that was used was retroviral insertional mutagenesis. The resulting tumorigenic variants that were obtained from WM35, expressed several phenotypic features characteristic of advanced-stage melanomas, including tumorigenicity in nude mice, and a significant increase in growth resistance to IL-1, IL-6- and TGFβ-induced inhibition in comparison with the parental WM35 cells (25).

The antiproliferation effect of these cytokines against melanoma cells has also been noted by other laboratories, e.g. the inhibitory effects of IL-1, TNFα, TGFβ, IL-6 and OSM on A375 melanoma cells (286,287,419), and the lack of response in other cell lines, e.g. MeWo (419). However, the authors of these studies paid little attention to the stage of disease progression the tumor cell lines were derived from, or no information was provided regarding the pathologic stage/origin of the cell lines used (as in the case of A375). Thus, there was no rational explanation for the variable response to the cytokines tested among the various human melanoma cell lines used for these studies (286,287,419,490). When the stage of tumor progression was taken into account, an increased resistance to IL-6 (383), OSM (383) and TGFβ (229) in advanced-stage melanoma cell lines was observed by others as well, in confirmation of our results.

It is known that IL-1, IL-6 and TGFβ can be produced by many types of cell, such as normal keratinocytes, macrophages, dermal fibroblastic cells, and endothelial cells, which together are the major cellular components in epidermal and dermal tissue compartments (217,274,306,335). Moreover, UV exposure can also induce IL-6 production in the skin (216), and tumor infiltrating leukocytes (TIL) have been found to be able to release a number
of cytokines, including TNFα and IL-6 (175). Thus, the slow and restricted (ie. dormant) growth of clinical early-stage melanoma cells can be accounted for, at least in part, by the "sensitive" phenotype of such tumor cells to the combined growth inhibitory effects brought about by multiple growth factors produced by adjacent stromal cells and normal cells (116). Some of these paracrine mediators may also be produced by endothelial cells present in melanoma because of angiogenesis. Production of IL-6 by endothelial cells has been detected (290), which may in turn help to restrict the growth of IL-6 sensitive early-stage primary melanomas (335). This hypothesis is supported by our results showing a progressive loss to the growth inhibitory effect of endothelium-derived regulators in human melanoma cells as a function of disease progression (335).

During the development of tumor progression, which involves multiple types of tumor-host interactions, some melanoma cell variants may eventually be selected for their ability to survive and proliferate in the dermis, which in turn may help them progress to a more malignant phenotype. While becoming resistant to growth inhibitory regulation, some of the selected melanoma cells may also begin to actually produce these same growth regulators. Thus, multicytokine resistance has been found to be associated with the expression and production of one or more of the growth inhibitory regulators (15,251). Production of IL-1 (36,62,68,225), IL-6 (68,86,237,251) and TGFβ (229,340,349) has been found in some of human melanoma cell lines that were derived from advanced-stage lesions. In addition, a study in fresh biopsy specimens showed positive expression of IL-6 in 14 out of 20 advanced-stage primary and metastatic human melanomas (80). Production of such negative growth regulators seems to be associated with the clinical stage of disease progression and depth of
tumor invasion (251,340). Recently, a growth inhibitory protein, called melanoma-derived inhibitory activity (MIA) was isolated from the culture medium of a metastatic melanoma cell line (44). Therefore, there is a good reason to speculate that the production of these growth regulators by advanced-stage melanoma cells could provide an additional source of paracrine growth inhibitors for the less malignant cells within a primary tumor mass, which in turn could lead the selective overgrowth of the resistant cells, i.e. their "clonal dominance" at the primary tumor site (211).

ii: Development of a resistant phenotype to multiple growth inhibitors detected in other human cancers

The resistant phenotype to growth inhibition by multiple paracrine growth factors which include TGFβ and various cytokines is also a feature observed in some other human cancers. In terms of proliferative responses to recombinant human IL-6, Serve et al. (377) examined 26 different human cell lines derived from a wide range of solid tumors, which included carcinomas of head/neck, lung, pancreatic, gastric, colorectal, renal, bladder, prostate, and ovary, as well as fibrosarcoma, glioblastoma, neuroblastoma, choriocarcinoma and two cell lines of human breast carcinoma (HTB22 and HTB19). Their results showed that none of the cell lines tested was affected by addition of IL-6 in vitro in terms of growth. Some other studies, on the other hand, have indicated that IL-6 is a potential autocrine growth stimulator for certain types of human solid tumor, such as renal cell carcinoma, ovarian carcinoma, invasive cervical carcinoma and prostate carcinoma (see also section in 5.2: IL-6 in human cancers). In terms of human breast carcinoma, there were several reports that have indicated strong growth inhibition mediated by IL-6 including such cell lines as T47D, MCF-7
and MDA-MB-415, but not others, e.g. MDA-MB-231 (73,92,311). However, further analysis suggested that these apparently discrepant results may be due to subtle differences in the relative malignant phenotype of cells tested. The biologically less aggressive breast carcinomas tend to be more sensitive to IL-6 mediated growth inhibition than their more malignant counterparts, similar to the situation we observed in cutaneous melanoma.

In another experimental model, rat liver epithelial cells were growth inhibited by both TGFβ and IL-6 in a dose-dependent manner, but tumorigenically transformed liver-derived cell lines showed some degree of resistance to both factors (179).

In human colorectal carcinoma, the sequential transition of tumor progression from adenoma to carcinoma was found to be accompanied by a reduced response to TGFβ induced growth inhibition (265). In normal keratinocytes, TGFβ is a potent inhibitor of cellular proliferation. TGFβ can also inhibit the growth of immortalized and nontumorigenic keratinocytes, but not of squamous carcinoma cell lines (55). Progressive abrogation of TGFβ controlled growth regulation was found in ras-transfected human keratinocytes expressing different levels of tumorigenic potential (122). The altered response to TGFβ was also shown in gliomas, in which low-grade tumors were found to be sensitive to TGFβ mediated growth inhibition whereas malignant glioblastomas were found to be growth stimulated (192), i.e., there is a 'switch' similar to what I observed in my melanoma studies using IL-6.

In addition to the acquired resistance to paracrine TGFβ-mediated growth inhibition, it is known that most cancers in situ and cancer cells in culture produce TGFβ. Such endogenous TGFβ may still function as a negative regulator of normal cell proliferation but cancer cells generally have the capability to overcome completely, or partially, this negative
regulation, by various mechanisms (465). TGFβ is also known as a modulator of the expression of other growth factors, such as PDGF, and the components of extracellular matrix (269,289,391). The ultimate effect of this endogenously produced TGFβ may be to stimulate cancer cells to invade, progress and proliferate by several mechanisms (204). Indeed, endogenous TGFβ can stimulate angiogenesis and stroma formation in vivo, and in so doing stimulate the growth of solid tumors.

The molecular changes that confer upon cancer cells a resistant phenotype to TGFβ-induced growth inhibition in malignant or advanced-stage of disease include loss of the type II TGFβ receptor (187) or mutations in the type II TGFβ receptor genes (267). In addition, overexpression of cyclin-dependent kinases, such as CDK4 can also make cells insensitive to TGFβ (181). In the case of loss of the receptor, the growth inhibitor activity of TGFβ could be restored by introduction of the type II receptor (187). Interestingly, addition of polyunsaturated fatty acids were also found to reverse the resistant phenotype in vitro in some type of tumor cells although the mechanism of this effect remains unclear (305,358).

From all of these studies it is obvious that increased resistance to various negative regulators of cell proliferation can contribute in part to the transformation and progression process of cancers.

iii): Multigrowth factor independence of advanced stage human cancers:

As discussed in the Introduction, deregulated response to growth stimulatory signals by paracrine or autocrine mechanisms is one of the accumulated cellular changes that would
lead the cell to an uncontrolled growth state. Many studies have indicated that cancer cells frequently have certain pathological alterations of their mitogenic signals, from the ectopic production of growth factors, abnormal function of growth factor receptors, altered signal mediators or transcriptional regulators of gene expression (3,64,180). Autocrine production and utilization of growth stimulatory factors have been considered to be one of the essential steps for cancer cells to be able to survive and proliferate independently. Many types of cancer cell display a production of autocrine growth factors. The number of such growth factors that tumor cells produce is generally associated with the stage of progression of the tumor. This conclusion comes from the fact that the more malignant a tumor cell population is, the fewer exogenous growth factors needed for their growth in vitro. In several cytokine-dependent cell lines, introduction and expression of the relevant cytokine encoding gene can give rise to the generation of growth factor independent transformants, such as IL-3, GM-CSF and IL-6 (209). Autocrine production of TGFβ was found to be associated with the transformed properties in human colon cancer cells (489), and a similar autocrine production of bFGF has been found in tumor cells, such as chondrosarcoma, melanoma, osteosarcoma, hepatoma, retinoblastoma and Kaposi's sarcoma (35,474).

In cells of the melanocytic lineage, several growth supplements to the culture base medium are required for sustained growth of normal human melanocytes, such as insulin, α-MSH, bFGF and TPA, whereas there is a reduced requirement in nevus cells and primary early-stage melanoma cells. In contrast, advanced stage tumors do not require any of these exogenously added substances to support their growth, and are able to grow well in essential medium even without fetal calf serum and added growth factors, after a period of adaptation
Consistent with such findings, experiments have indicated the absence of autocrine growth factor production, but the presence of the specific receptor for such growth factors in normal human melanocytes. Constitutive production and utilization of bFGF and MGSA has been found in benign nevus cells (148,343,379), whereas it has been shown that metastatically-competent melanomas are able to produce several different growth factors and cytokines; these include bFGF, MGSA, IL-1, NGF, PDGF, TGFα, TGFβ, IL-6 and IL-8 (37,251,276,343,361). The number of growth factors produced by melanoma cells of early-stage lesions lies somewhere between that of nevus cells and metastatic melanoma cells, and include bFGF, MGSA and IL-8 (37,148,343). Some of these growth factors, eg. bFGF, MGSA, and IL-8, have been shown to have an autocrine growth stimulatory role, directly or indirectly, with respect to melanoma cell growth (148,343,361).

In contrast, the functions of some other growth regulatory factors found in melanoma cells remain unknown; these include LIF and IL-11 (317).

Besides the production of more and greater levels of growth factors, advanced-stage melanomas are growth resistant to paracrine growth inhibitors, such as IL-6, and moreover, become competent to produce and utilize it as a growth stimulator by various possible mechanisms. Such a transition from growth inhibitor to growth stimulator occurs as tumor cells progress toward metastatic competence. The precise mechanisms responsible for this transition in growth factor responsiveness are unknown. Tumor suppressor gene products, such as p53 and Rb were found to be able to repress the IL-6 gene expression (357), while mutant N-ras oncogene can change cells from an IL-6-dependent to an IL-6-independent growth state (39). Whether the abnormalities of p53, Rb and ras are responsible for IL-6
functional transition in normal and malignant melanocytic cells require further studies. Some experimental studies have shown an association of p53 and/or Rb with deregulation and autocrine stimulation of IL-6 in leukemia cells and prostate cancer cells (145,488). The autocrine stimulatory role of IL-6 has been considered to be a direct effect on cellular proliferation based on evidence obtained from experiments using neutralizing antibodies or antisense oligonucleotides in several other systems of human cancer, eg. renal cell carcinoma, Kaposi's sarcoma, prostate carcinoma cells, multiple myeloma and lymphoma (209,277,279,382,424,451).

However, in human advanced-stage melanoma positive for endogenous IL-6 production, we had difficulties to confirm the existence of an autocrine stimulation or inhibition in cell culture in vitro by using neutralizing antibodies or after long-term transfection with cDNA vector expressing IL-6 antisense mRNA (see Chapters 2 and 3). The results from experiments with oligonucleotides, in which sense and antisense oligonucleotides were tested in both IL-6 producing and non-producing melanoma cells, certainly implied a possible autocrine loop of growth stimulation for IL-6 activity (Chapter 2). However, we now feel caution is warranted in explaining and interpreting the data generated from such antisense oligonucleotide experiments, as also noted recently by many others (after our data in Chapter 2 has been published) (395-397) (see also the discussion section of Chapter 3). Recent attempt in separate experiments to add exogenous IL-6 failed to rescue the growth inhibition induced by the antisense IL-6 oligonucleotide preparations. The question whether IL-6 has an unequivocal autocrine role of growth stimulation in human melanoma cells remains to be answered by using modified oligonucleotides of different antisense sequences, or neutralizing...
antibodies against IL-6 receptors (or antibodies from different sources). Nevertheless, our work of stable transfection with an antisense IL-6 expression vector revealed that there is ultimately a significant growth stimulatory role in vivo in tumor growth by IL-6 in some advanced-stage melanoma cells positive for endogenous IL-6. The mechanism of this effect remains unknown, but some kind of paracrine tumor-host interaction is probably involved.

A transition from a paracrine growth inhibitor to a growth stimulator during disease progression has also been reported for TGFβ. Some cancer cell lines display a degree of resistance to TGFβ-induced growth inhibition, while in other cases the growth of cancer cells is actually stimulated by exogenously added active TGFβ. Such growth stimulatory activity of TGFβ is considered by some to be an indirect effect mediated through up-regulation of other growth stimulatory factors, such as PDGF, and VEGF (192,204,289). The autocrine activity of TGFβ has been found in many types of cancer cell, and experiments using antisense approaches or neutralizing antibodies have indicated that such endogenously produced TGFβ can act as either a growth inhibitor or a growth stimulator for producing cell in vitro, depending upon the type of the cells tested (178,229,465).

5.2. The biology of interleukin 6 and its family members:

i): The biology of IL-6:

Interleukin (IL) 6 is one of the most important pleiotropic cytokines, and is involved in immune responses, acute phase reactions, haematopoiesis, host defense mechanisms and tumor progression. Through its diversified biological activities, IL-6 was also known at one
time as β2-interferon, B-cell stimulatory factor 2, 26 kDa protein, hybridoma / plasmacytoma growth factor, hepatocyte stimulating factor and monocyte granulocyte inducer type 2. The molecular cloning work later proved that these activities are all mediated by the same molecule, renamed IL-6 (168,217,434).

The cDNA for human IL-6 predicts a precursor protein of 212 amino acids including a hydrophobic signal sequence of 28 residues and two potential N-glycosylation sites and four cysteine residues (169). There is 65% homology at the cDNA level and 42% homology at the protein level between human and murine IL-6 (217,434). Murine IL-6 cDNA encodes a protein of 211 amino acids with a hydrophobic signal sequence of 24 residues. In spite of some sequence divergence, human IL-6 is biologically active on mouse cells whereas murine IL-6 does not activate human IL-6 receptors, i.e. is biologically inactive against human cells (434).

The human IL-6 gene is located on chromosome 7 (7p21) (372) and consists of five exons and four introns with the same overall exon/intron pattern as the gene for granulocyte-colony-stimulating factor (G-CSF) (472). The murine IL-6 gene has been mapped to the proximal region of chromosome 5 (283). IL-6 gene expression is enhanced by infection with different RNA- and DNA-containing viruses (50,372) and by inflammation-associated cytokines such as IL-1, TNF (232,427), PDGF, and interferons (209,372), whereas glucocorticoids usually down regulate the expression of IL-6 (52,216). CD40 ligand or monoclonal antibody against CD40 can also induce the expression and production of IL-6 in multiple myeloma cells (429,459). Analysis of the 5'-flanking region of the IL-6 gene has revealed a complex array of regulatory sequences with several positive and negative elements,
including a glucocorticoid responsive element (GRE), an AP-1 binding site, a c-fos serum responsive element (SRE), a cyclic AMP responsive element (CRE), and a NF-kB-binding site \(97,168,191,372\). A 23 bp multi-response element (MRE) has been identified within the c-fos SRE domain and may be involved in IL-6 induction by IL-1, TNF, forskolin and phorbol ester \(168,191,372\). In functional assays, dexamethasone-activated glucocorticoid receptors strongly repress the activity of the IL-6 functional element, MRE \(372\).

One of the functions initially identified for IL-6 was the induction of terminal maturation of B cells that is essential for antibody producing plasma cells. IL-6 can increase the production of IgM, IgG, and IgA in PMA-stimulated peripheral mononuclear cells without stimulating B-cell proliferation \(169,217,434\). An involvement of IL-6 in T-cell activation was also discovered independently by several investigators working in different areas. The consensus that has emerged from these studies is that IL-6 is an essential competence factor that synergizes with IL-1 to control the initial steps in T-cell activation. Part of this synergy stems from the fact that IL-6 acts predominantly to enhance IL-2 responsiveness, whereas IL-1 stimulates IL-2 production \(217,434\).

IL-6 is also involved in haematopoiesis, in which it acts synergistically with IL-3 on multipotent progenitors, apparently by reducing the \(G_0\)-residence time of the haematopoietic stem cells \(217,434\). Independently, IL-6 was also found to act synergistically with GM-CSF and M-CSF on progenitor cells \(65,341,434\). Such an effect may provide a potential use for IL-6 in bone marrow transplantation \(158,159,217\). In addition to effects on the proliferation and differentiation of normal precursor cells, IL-6 has also been shown to induce macrophage and granulocyte macrophage \textit{in vitro} from progenitor cells and to inhibit cell
growth in both human and murine myeloid leukemic cell lines, eg. U937 and M1 (217).

One other interesting aspect of IL-6 is its function as a survival factor, by virtue of its ability to suppress the process of programmed cell death, or apoptosis. For example, in myeloid leukaemic M1 cells, the apoptotic process induced by wild-type p53 was found to be inhibited by exogenous IL-6 (477, 478). This IL-6-mediated anti-apoptotic process is probably mediated by regulating and balancing various oncogene or proto-oncogene products that are known to be involved in influencing apoptosis, such as bcl-2, bcl-\(X_L\) and bax (250, 369). IL-6 may also be able to stimulate the expression and production of VEGF which is known to be a major mediator of angiogenesis (85).

Infections, injuries, malignant tumors, and a variety of immunological disorders trigger a complex physiologic response in the organism known as the "acute phase response". IL-1 and TNF were the first cytokines identified that 'travel' from the site of injury to the liver where they trigger the hepatic acute phase reaction. However, neither of these factors was able to induce the full spectrum of acute phase proteins that normally follows exposure to crude monocyte supernatants. The missing active protein, termed "hepatocyte-stimulating factor", turned out to be identical to IL-6 (125, 434). IL-6 levels increased rapidly after infection, ranging from 5 to 100 ng/ml in the peripheral circulation, and >500 ng/ml in localized infected compartments (372, 434). Since then, the importance of IL-6 as an inducer of the acute phase response has been confirmed by the fact that IL-6 induces acute phase proteins \textit{in vivo} and stimulates the full spectrum of acute phase proteins in adult human hepatocytes (217, 434). The molecular entity that mediates this intracellular response in the IL-6 signalling pathway has been identified, and was termed the "acute phase response
factor", or APRF, also referred to as Stat3 (5,452,487) (see below).

ii) IL-6 receptors and the signalling pathways:

IL-6 receptor activity is achieved by two cell surface transmembrane proteins, the IL-6 receptor (gp80 with high IL-6 binding affinity) and a signal transducing protein, called gp 130 (218). The IL-6 molecule has a high binding affinity for the gp80 subunit of IL-6 receptor with a single class of binding sites (304,351). The extracellular region of human gp80 consists of an Ig-like domain and a cytokine receptor family domain that comprises two fibronectin type III modules (30,469,473). Surprisingly, the cytoplasmic domain of gp80 is relatively short and does not seem to be involved directly in signal transduction. Further studies have revealed that binding of IL-6 to receptor gp80 triggers the extracellular association of gp80 with a non-IL-6 binding signal transducing protein, gp130, which in turn generates the IL-6 signal (165). Gp130 alone does not have binding activity for IL-6 but is required for high binding affinity of IL-6 with gp80 (165,218). The extracellular region of gp130 has six fibronectin type III modular repeats, and part of this region of about 200 residues has features that are typical of the cytokine receptor family (30,165). The cytoplasmic region was shown to be essential for generating intracellular signals (294,467).

Activation of the gp130 protein triggers a series of intracellular signalling pathway responses, which includes (but is not limited to) two distinct signalling systems, the "Jak-Stat" pathway and the MAP kinase pathway (219). Jaks, or Janus kinases are another subfamily of cytoplasmic protein tyrosine kinases, or "Just Another Kinase", which mediate the signal transduction of several cytokine receptors, such as IL-2, and IL-6 (186). Members of the
Jak family include Jak 1, Jak 2, Jak 3 and Tyk 2; their expression is usually ubiquitous with the exception of Jak 3 (186). The specific binding of ligand to its receptors results in the tyrosine phosphorylation and activation of Jak kinase catalytic activities (186). It has been shown that IL-6 and other members of IL-6 family such as ciliary neurotrophic factor and oncostatin M can induce the phosphorylation of the Jak1, Jak2 and Tyk 2 kinases (in certain cells such as EW-1 cells and HepG2 hepatoma cells) (186,257,393). The activation of Jaks causes the phosphorylation of tyrosine residues of certain DNA binding proteins, called Stat (signal transducers and activators of transcription). IL-6 triggers tyrosine phosphorylation to activate Stat3, also called APRF (acute-phase response factor) and Stat1. Following phosphorylation, the STATs form dimers through the intermolecular association of the SH2 domains, resulting in the translocation to the nucleus, where the STAT dimers bind response elements to regulate downstream expression of such target genes (5,185,257,452,487). Such genes so far identified include junB (82), c-jun (362), α2-macroglobulin (5), and p21waf1 (77). This is consistent with the ability of IL-6 to promote cell growth, induce acute phase response and inhibit cell proliferation in various circumstances.

Another pathway for IL-6 signalling may involve the MAP kinases, in which the activated MAP kinase catalyzes the phosphorylation on threonine of a nuclear transcriptional factor, namely NF-IL6 (219). In an IL-6 dependent myeloma cell line, called ANBL6, mutant N-ras endowed the cell line with an IL-6-independent growth status (39). In cervical carcinoma-derived cells, IL-6 was found to stimulate cellular proliferation. Neutralizing antibody to the EGF receptor inhibited IL-6-induced growth stimulation (184). In the cell line AF-10, cloned from human IgE myeloma cell line U266, stimulation of cell proliferation by
IL-6 was found to be accompanied by the induction of MAP kinase (mitogen-activated protein kinase) activities as determined by \textit{in vitro} phosphorylation of microtubule-associated protein-2 (MAP-2) (90). In Kaposi's sarcoma-derived cells, oncostatin-M, which also utilizes gp130 as a signal transducing protein, induced the phosphorylation and activation of MAP kinase (13). Moreover, recent studies have shown that the two signal pathways, ie. Jak-STAT and \textit{ras}-MAP kinase have certain interactions at the MAP kinase level upon stimulation by interferons (94). Activated \textit{src} oncogene may also be able to induce the tyrosine phosphorylation of Stat3 (480). Furthermore, it is also shown that IL-6 can induce the phosphorylation of other non-receptor tyrosine kinases, such as Fes, Btk and Tec (271).

In addition to Stat protein, a nuclear transcription factor, NF-IL6 regulates not only the expression of IL-6 gene but also the biological activities of IL-6 (218,219). It was identified initially through its activity in inducing IL-6 gene expression by IL-1. Molecular cloning revealed that NF-IL6 cDNA has a high degree of similarity to a liver-specific transcription factor, C/EBP (218). Analysis of IL-6 promoter sequences indicated the specific binding site for NF-IL6 (168). Interestingly, both expression and function of NF-IL6 are tightly regulated by IL-6. NF-IL6 can bind to IL-6 responsive elements of several positive acute phase protein genes (168).

It has been shown that IL-6 can induce the response of some 'immediate' genes, such as \textit{c-myc}, \textit{junB} and \textit{c-myb}, which may be responsible for some but not all IL-6 activities (171,172,342). Constitutive expression of \textit{c-myc} could partially block IL-6-induced growth inhibition and terminal differentiation in M1 cells (171,172). Although treatment with TGF\(\beta\) and IL-6 can suppress pRb phosphorylation, the decrease in \textit{c-myc} gene
expression induced by IL-6 appears not to be associated with the suppression of pRb phosphorylation in the M1 cell line (342). Another report has indicated that such terminal differentiation and growth arrest induced by IL-6 was also associated with an increase in protein-tyrosine-phosphatase activity and a decrease in tyrosine phosphorylation of cellular protein (481).

Given what is known about IL-6 receptors and intracellular signal transduction pathways so far identified, it is clear that the ultimate biological activities triggered by gp130 are obviously dependent upon cell type and cross-talk with other factors since IL-6 induces diverse biological activities, including growth stimulation, growth inhibition, differentiation and inhibition of apoptosis. In human melanoma cells, activation of gp130 by different ligands, such as IL-6, oncostatin M, leukemia inhibitory factor, or IL-11 induces a significantly different response of cell growth although they all share the same gp130 as signal transducing molecule (251,253,317).

A number of attempts have been made to discover a possible alteration, or defect, in IL-6 signalling pathways which could explain the resistance to IL-6 induced growth inhibition, or for the transition from growth inhibition to growth stimulation, in human melanoma cell lines. Early studies on IL-6 receptor gene expression, receptor-ligand binding activities, analysis of tyrosine phosphorylation status of Jak kinase and Stat DNA binding activities to the promoter of α2-macroglobulin did not reveal any consistent alteration that could explain the IL-6 resistant phenotype or the stimulatory effect of IL-6 in advanced-stage melanoma cell lines (see Chapter 2, and our unpublished results). These 'negative' results eventually led to a decision in our laboratory to analyze the complex cell cycle control machinery in these
cells. A postdoctoral fellow Dr. V.A. Floerenes under the joint supervision of Dr. Kerbel and Dr. Joyce Slingerland recently obtained results which have implicated the involvement of the two 'universal' cyclin dependent kinase inhibitors (CDKI), p21\textsuperscript{WAF1} and p27\textsuperscript{K1P1} (115). For example, IL-6 treatment of WM35 cells results in up-regulation of p21\textsuperscript{WAF1} whereas variants of WM35 selected for tumorigenic competence in nude mice express much less p21\textsuperscript{WAF1} (115). DNA binding assays have indicated an IL-6 inducible binding of Stat to p21\textsuperscript{WAF1} promoter in WM35 cells (Lu, C. unpublished observation). These results are consistent with the recently proposed notion that p21\textsuperscript{WAF1} may be a suppressor of malignant melanoma metastasis, or progression. This stems from evidence that p21\textsuperscript{WAF1}, when cloned independently as the melanoma differentiation associated gene called mda 6, has a much higher expression in normal melanocytes than metastatic melanomas, and is also higher in early stage, or early stage-like than progressed melanomas (194,458). Experiments to understand the role of p21\textsuperscript{WAF1} in the transition of IL-6 activities in malignant melanoma are being undertaken in this laboratory by analyzing the DNA binding activities of activated Stat3 to the p21\textsuperscript{WAF1} promoter and by up- or down-regulating the expression of p21\textsuperscript{WAF1} gene in melanoma cell lines using genetic technology.

iii) Other members of the IL-6 family:

With respect to other cytokines, IL-6 has a significant homology at the cDNA level with leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), G-CSF, and myelomonocytic growth factor (29,217,263), which comprise a family of cytokines, called the IL-6 family. In terms of ligand receptor interactions, LIF, OSM
and CNTF have a closer relationship with IL-6 since they all can use the same molecule, i.e. gp130, as part of the receptor complexes and/or signalling transducing protein to trigger or mediate intracellular signals. This probably explains some of the overlapping biological activities among these cytokines (218).

LIF (leukemia inhibitory factor), like IL-6, is a highly pleiotropic cytokine and is produced by many types of cell; production of LIF has been detected in human bone marrow stromal cells, thymic epithelial cells, keratinocytes, activated monocytes and certain tumors, which include bladder, squamous and pancreatic carcinoma, melanoma, breast adenocarcinoma, and monocytic leukemia (137,167,230). LIF can inhibit the differentiation of embryonal stem cells, induce the terminal differentiation of M1 cells and stimulate acute phase protein synthesis in hepatocytes, which overlaps with some, but not all of known IL-6 activities (59,137,167,230). The action of LIF is initiated by the binding of ligand to the LIF receptor, a structurally gp130-related membrane protein (128). Binding of LIF to its receptor causes the heterodimerization of gp130 and LIF receptor, which subsequently triggers intracellular signalling by phosphorylation (95). Despite the fact that LIF uses gp130 for signal transduction and LIF can reproduce some of the functions of IL-6 (such as induction of differentiation of M1 myeloid leukemia cells) (59,95,218,375), we have found that LIF is not able to reproduce the growth inhibitory activity of IL-6 in human melanoma cells (59,253), implying the existence of a LIF signalling pathway (in human melanoma cells) distinct from IL-6.

OSM (oncostatin M) was initially isolated as a growth inhibitor of A375 human melanoma cells from serum-free supernatants of U937 cells that had been treated with PMA
(57,263,484). The cDNA sequence of OSM predicts a protein comprising 252 amino acids and the human OSM gene is localized to chromosome 22 (263,350). It can mimic most if not all IL-6 biological activities, such as inducing terminal differentiation and growth inhibition of M1 cells (59,350,484), and acting as an autocrine growth stimulator in AIDS-Kaposi's sarcoma cells (278,297). It has been shown that OSM binds to gp130, and to the LIF receptor, both of which are components of the receptor complex for OSM (126,127,246,262). The common biological activities of OSM with IL-6 are probably the result of using the same signalling transducing pathway mediated by gp130. Neutralizing monoclonal antibody against gp130 can abrogate the stimulatory activities of OSM in human plasmacytoma cells (310). In human melanoma cells there is a significant increase in growth resistance to OSM-induced inhibition observed in advanced-stage cell lines. However, we have found (as indicated in Chapter 4) that there is a differential effect between OSM and IL-6 in human melanoma cell lines which clearly suggest the existence of distinct signal pathways for OSM and IL-6, in addition to their common pathways.

Another member belonging to the IL-6 family that utilizes gp130 in its receptor complex is CNTF (ciliary neurotrophic factor) (95,188,189,218,394). The binding of CNTF to its receptors results in the formation of a complex with the LIF receptor and gp130, which leads to the activation of an intracellular phosphorylation cascade (95). Besides members in this family, IL-11 and cardiotoxin 1 have many IL-6-like biological activities and also use gp130 as part of their receptor complex to induce intracellular signals (219,471,479). However, we have not found any significant effect of IL-11 on the growth of human melanoma cells associated with either early or advanced stages of malignancy
iv): The possible role of IL-6 in other human malignancies:

Besides the effects on human malignant melanoma cells, IL-6 has been found to have significant effects on the growth of many types of human cancer. A growing literature has indicated an important possible role for IL-6 in the development of advanced-stage disease in both haematopoietic and solid malignancies.

In human haematopoietic malignancies, the main effect of IL-6 on cellular proliferation is, in general, a stimulatory one. Initial studies indicated that mouse plasmacytomas required IL-6 for cell growth; removal of IL-6 resulted in a G1 growth arrest in IL-6-dependent plasmacytomas (434,442). Subsequent observations have extended this to human multiple myelomas. It was shown that freshly isolated myeloma cells from patients express IL-6 gene and produce IL-6 protein, as well as IL-6 receptors. Addition of neutralizing antibodies against IL-6 or IL-6 receptors was found to inhibit the proliferation of the cells, indicating the existence of an autocrine growth stimulatory role of IL-6 in such cells (208,310). Such an autocrine stimulatory activity of IL-6 for myeloma cells was quickly confirmed by many independent investigators by the use of neutralizing antibodies (359) and/or antisense oligonucleotides to the IL-6 gene (240,366). Some human myeloma cell lines require IL-6 from paracrine sources for their growth and survival prior to acquisition of autocrine production (193); transfection with an IL-6 gene expression vector could lead to a change from an IL-6 dependent to an IL-6 independent state and a more malignant phenotype (316). Mutant N-ras oncogene expression can also endow myeloma cells with an IL-6-
independent growth phenotype (39). IFN-α was found to induce growth stimulation in human myeloma cell lines and this was found to be mediated by induction of autocrine IL-6 production since neutralizing antibodies against IL-6 inhibited IFN-α induced growth stimulation (198). IL-6 was found to act as an autocrine growth stimulator in human lymphoma cell lines (475) and it has been detected in some types of human lymphoma when lymphoid tissues were examined (177). In addition to its own direct proliferative modulation, IL-6 also interacts with other growth factors and cytokines to stimulate growth, or enhance the responsiveness to other growth modulators, in human myeloma cells (170).

The effect of IL-6 in the development and progression of human solid tumors is a more complex issue compared to haematopoietic malignancies. The "dual" role or effect of IL-6 in the development of human cutaneous melanomas has been discussed in previous sections of this thesis (see Chapter 1, Chapter 2, Chapter 3 and section 5.1). The discussion below will focus on non-haematopoietic solid tumors (other than human melanomas).

In terms of anti-proliferative effects, IL-6 has been shown to affect the growth of about 50% of human breast carcinoma cell lines thus far examined, while the remainder were found unresponsive to exogenously added IL-6. This appears to correlate with oestrogen receptor status (in breast cancer cells) and relative malignant status of the cells (73,78,92,311,322). Breast cancer cells sensitive to IL-6 induced growth inhibition are generally oestrogen receptor positive, whereas IL-6 resistant cells are oestrogen receptor negative, and show positive endogenous IL-6 production (79). The same is also true in human prostate cancer cell lines; no IL-6 protein was detected in cell lines responsive to androgen, whereas 4 out of 5 androgen independent cell lines were found to produce IL-6 (426). Such
an association of IL-6 responsiveness and IL-6 production with hormone responsiveness and
dependence may, like melanoma, reflect a possible transition of IL-6 activity in these types of
cancer during disease progression. Again, this could be an indication of a contribution of
exogenous or endogenous IL-6 in solid tumors to their malignant aggressiveness.

In human non-small cell lung cancer cell lines, IL-6 acts as a growth inhibitory factor
by both a paracrine and an autocrine mechanism. However, a decreased sensitivity of
malignant cells was found compared with that of normal bronchial epithelial cells (409).
Such IL-6 induced growth inhibition was not found in the same study in small cell lung cancer
(SCLC) cells (409). The work by Serve et al. showed that none of the 26 different human
cancer cell lines tested was growth "responsive" to exogenously added IL-6 (377). The
same was true in cells from human small cell lung cancer, gastric carcinoma, glioblastoma
multiforma and two cell lines from breast carcinomas, as reported by Pedrazzoli et al.
(322). Some other studies have indicated a paracrine growth stimulatory effect of IL-6 on
cells of colorectal carcinoma cell line (233), chondrosarcoma cell (142) and mammary
epithelial cells transfected with the int2 gene (28). In cervical carcinoma-derived cell
lines, addition of IL-6 or IL-6 soluble receptor stimulated cell growth in vitro, which was
accompanied by an increase in TGF-β and amphiregulin. Blocking antibodies against EGF
receptor reduced IL-6-stimulated cell proliferation (184).

A number of studies have indicated that a broad spectrum of human solid tumors
produce IL-6. Expression of IL-6 was observed by immunostaining in several human tumors,
such as squamous cell carcinoma, mammary adenocarcinomas, and carcinomas of colonic,
ovidian and endometrial origin (405). In human glioblastoma (433) and renal cell carcinomas
IL-6 production has been detected both in *in vitro* cultured cells and in tumor cells of tissue sections from patients by immunohistochemical analysis. However, there are other cases where IL-6 in tumor specimens has been found to be exclusively associated with infiltrating stromal cells, such as intra-tumoral macrophages in cancer of the cervix (418). In some cases, evidence has been obtained to suggest that IL-6 acts as an autocrine growth stimulatory factor. Among the well-documented tumors are Kaposi sarcoma-derived cells (279), prostate cancer cells (145), renal cell carcinoma cells (227,277), and cervical carcinomas (109,184). In contrast, in the case of lung cancer cells, endogenously produced IL-6 may still act as a growth inhibiting factor (409).

Autocrine production and growth stimulation of IL-6 has been found in a large number of cell lines derived from human renal cell carcinoma (124,141,277,408). Neutralizing antibodies against IL-6 were found to inhibit the growth of these cell lines (141,277). Freshly isolated renal cell carcinomas expressed mRNA and produced IL-6 protein (277,408). The anti-proliferative effect of IFNγ could be abrogated by addition of exogenous IL-6, implying that an indirect effect of IFNγ on renal cell carcinoma cell growth was operative by inhibition of autocrine IL-6 (141). Production of IL-6 by cancer cells is considered by some investigators to be responsible for some of the paraneoplastic syndromes of patients with renal cell carcinoma (424). IL-6 production was also found in human prostate cancer cells; addition of antisense IL-6 oligonucleotides, or neutralizing antibodies against IL-6 into cell cultures resulted in a significant growth inhibition of the cells, suggesting an autocrine growth stimulatory role for IL-6 in this type of cancer (48,145,382).

Some experiments have detected positive gene expression and protein production of
IL-6 and/or IL-6 receptor in tumor cells but there was a lack of evidence to indicate a growth regulatory role for this endogenous IL-6. Examples include human squamous cell carcinoma cell lines (320), glioblastoma cells (433), ovarian tumor cells (313,451), mesothelioma cells (364), hepatocarcinoma cells (22), colon carcinoma, breast carcinoma and neuroblastoma (79,124).

Besides an effect on cell proliferation, IL-6 can also act as a survival factor for some cancer cells. Inhibition of programmed cell death was implicated on the basis of suppressed apoptosis being observed after addition of IL-6 to cells undergoing differentiation (210), or apoptosis induced by various conditions, such as serum starvation (242), exposure to cytotoxic compounds (249), or withdrawal of IL-6 from IL-6-dependent cells (245,354,369). In M1 cells, the apoptotic process induced by wild-type p53 can be inhibited by IL-6 (477,478). Such anti-apoptotic activities are probably mediated by regulating and balancing the expression (and perhaps the function) of bcl-2, bcl-XL and bax gene products, which are essential regulators of the apoptotic process (250,369). It also seems that the process of IL-6 inhibition of apoptosis requires de novo protein synthesis (245). The underlying molecular mechanism that is responsible for delivery of both growth and survival signals is not yet fully understood, but some experimental results have implicated the involvement of oncogenes, such as ras, since oncogenically activated N-ras can change the cell growth requirement from an IL-6-dependent to an IL-6-independent state (39). Furthermore, activated ras oncogenes may be able to inhibit programmed cell death (338). In the case of some tumor cells that produce endogenous IL-6 as an autocrine growth factor, it has been shown that exposure of the cells to IL-6 neutralizing antibodies can make them more sensitive to certain toxic
chemotherapeutic drugs, such as cis-diamminedichloroplatinum (48,282). Therefore, *endogenous* production of IL-6 could, in theory, provide such cells not only with a growth advantage but also a survival advantage under conditions that induce apoptosis.

IL-6 also has some effect on cell-cell interactions. In breast cancer cells, IL-6 was found to decrease the association between cells, resulting in an enhanced motility of cancer cells, i.e. it acted as a "scattering factor" (373,410,411). The adhesion molecules responsible for such a dissociation have not been identified as yet. It has also been shown that IL-6 can up-regulate ICAM-1 in human melanoma cells (215).

High levels of IL-6 in the blood circulation have been found in patients with both haematopoietic and nonhematopoietic cancers. Although the precise source of IL-6 production is not clear, it is generally believed that such high levels of IL-6 are responsible, at least in part, for some of the systematic abnormalities in cancer patients, such as cachexia (398,414), hypercalcemia associated with squamous carcinoma (476) and onset of fevers (16,424). Experimental data seem to support the clinical observation that IL-6 is involved in cancer related cachexia (388,412). The serum IL-6 levels in patients with benign diseases were the same as in normals, but much higher in patients with malignant tumors (53% of primary epithelial ovarian cancer and 100% with breast cancers) (360,436). In patients with epithelial ovarian cancer, the serum levels of IL-6 were found to have a good correlation with tumor burden, clinical disease status and patient survival; patients with higher IL-6 levels in their serum had shorter survival times (38,360,417). The source of such high levels of IL-6 may derive from both cancer cells and various host cell types (313). A similar correlation of decreased survival times with high serum IL-6 levels was also found in patients
with Hodgkin's disease (231), or melanomas (416). Analysis of blood and urine levels of IL-6 suggested that removal of the bladder carcinomas resulted in a marked decrease of IL-6 levels (371).

The observation of such high levels of IL-6 in the blood circulation of patients with advanced cancers and the enhancing effect of IL-6 on the growth of some human solid cancers would seem to contra-indicate the notion of administrating exogenous recombinant IL-6 to cancer patients in order to boost their immune systems against cancer. This may be different from IL-2 dependent immunotherapy, since IL-2 levels are generally low in patients with advanced cancers (107). Nevertheless, there are some reports of positive IL-6 immunotherapy studies (ie tumor regressions) using murine B16 melanoma (402), mouse fibrosarcomas (293) and the Lewis lung carcinoma (207,314,331). In such studies, cells transfected with IL-6 cDNA expression vectors formed smaller tumors and with less metastatic aggressiveness, in syngeneic, immunocompetent mice compared to their parental cell lines (293,331,402). However, if the loss of body weight of the mice, which may be a result of high IL-6 in circulation, is taken into consideration, the tumor growth rate between the two groups is not different in the final analysis (314). A study by Armstrong et al. using B16-derived murine melanoma cell lines showed that transfection with an IL-6 expression vector greatly reduced tumor growth in both syngeneic and nude mice, indicating an direct growth inhibitory effect of IL-6 on tumor cells in vivo (18). There is also the issue of the potent immunogenic properties of the mouse tumors used in such studies which makes them much more vulnerable to the (boosted) immune system. The relevance of such models to human cancers is dubious, as discussed in Chapter 3.5.
In summary, I have found during my Ph.D. thesis studies a "multicytine resistant" phenotype that develops in advanced-stage human melanoma cells with respect to the growth inhibition induced by several cytokines, such as IL-1, IL-6, TNFα, and OSM. In particular, there is a transitional change in IL-6 function and effects in human melanoma cells during disease progression from early- to advanced-stageS. Analysis of IL-6 receptors and signal transducing pathways implicate a possible alteration of Stat3 function and its target gene expression, which may be involved in the development of resistance to IL-6 induced growth inhibition observed in advanced melanoma cells.

5.3 Possible future experiments:

During my Ph.D. study, I have accomplished some work as described in Chapters 1 to 4, as well as raised more questions. The followings are some of the possible projects that I would like to pursue and may lead to a better understanding of the role that IL-6 and other related cytokines may play in the development and progression of human cancers. In fact, efforts have been made to initiate studies during the writing of this thesis by myself and others in the laboratory.

1. Analysis of IL-6 gene expression by in situ hybridization or protein production by immunohistochemistry in human melanomas:

Most studies on IL-6 gene expression in human melanomas thus far have utilized only established cell lines, and these have indicated that about half of the (advanced-stage) melanoma cell lines express IL-6. This could, however, be a tissue culture dependent
phenomenon or artifact. In order to link the experimental findings with the clinical (\textit{in vivo}) situation, it is necessary to determine whether human melanoma tumor cells, both early-stage and advanced-stage, express the IL-6 gene and produce IL-6 protein \textit{in vivo}. In this regard a recent report has indicated that tissue biopsies from both advanced-stage primary and metastatic melanomas express the IL-6 gene, as analyzed by a PCR technique. However, IL-6 gene expression was not localized at the cellular level because of the presence of stromal cells along with melanoma cells (80). Future work should involve an analysis of IL-6 gene expression by \textit{in situ} hybridization, and IL-6 protein production by immunostaining techniques. Polyclonal antibodies are available for this purpose and have in fact been used in studies with other human cancers, such as squamous cell carcinoma, ovarian carcinoma, mammary or colorectal adenocarcinomas (405).

2. Involvement of p53 in IL-6 expression and function:

A link between IL-6 and the p53 tumor suppressor gene appears to be getting closer because of a number of independent studies indicating some sort inter-relationship between the two. For example, IL-6 was found to be able to inhibit apoptosis of myeloid leukemic cells induced by wild-type p53 (478). In another study it was suggested that the wild-type p53 and Rb tumor suppressor genes could repress IL-6 gene expression (357). These observations may link the tumor suppressor genes, p53 and Rb, with the deregulated expression of IL-6 in melanomas. Some studies have indicated that p53 mutations are not common in melanomas as assessed by DNA sequencing methods (101,445), but expression of "dysregulated" p53 protein is common in advanced stage human melanomas, as analyzed by immunohistochemical staining methods (236,399). It has been suggested that the p53 protein in these situation may
not function properly to regulate its downstream gene expression, such as p21\textsuperscript{WAF1} (21). More recently, IL-6 gene expression was found to be associated with inactivation of the p53 and/or Rb suppressor genes in leukemia cells and prostate carcinoma cells (145,488). Another link between IL-6 and p53 is through the CDK inhibitor p21\textsuperscript{WAF1} since it is well known that the expression of p21\textsuperscript{WAF1} is under the control of p53, and more recently was shown to be regulated by Stat3, and Stat1 as well (77,135). IL-6 can also inhibit wild-type p53-induced apoptosis. For all the reasons, it would seem appropriate to investigate the possible abnormalities of p53 and/or Rb in the context of the switch of IL-6 function and gene expression in human melanoma cell lines as a function of disease progression.

3. Search for genes and proteins that are potentially involved in mediating IL-6 signalling of growth inhibition:

Most, if not all, the published work on IL-6 regulated genes and proteins has been carried out in haematopoietic cells, in particular the murine myeloid leukemia cell line M1. In M1 cells, IL-6 induces both terminal differentiation and growth inhibition, which was found to be mediated partially by suppression of c-myc, and c-myb gene expression, and activation of protein-tyrosine-phosphatase activity (171,172,481). The down-regulation of c-myc and c-myb gene expression appears to be essential for M1 terminal differentiation, but not for IL-6 mediated growth inhibition (171). In contrast, the increased activity of protein-tyrosine-phosphatase(s) appeared not to be specific for growth inhibition since TGF\(\beta\)-induced growth inhibition in M1 cells did not change the activity of protein-tyrosine-phosphatase (481). On the other hand, LIF is as potent as IL-6 in M1 cells in terms of inducing terminal differentiation and growth inhibition, but does not induce any changes with respect to cellular proliferation in human melanocytic cells (59,171,172,253). It seems evident that such
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studies have provided useful information regarding possible mechanism of IL-6 action in M1 cells, but this information may be of limited value with respect to the effects and functions of IL-6 in the melanocytic lineage. In this respect, preliminary studies in our laboratory have excluded a direct involvement of the DNA binding activity of APRF, or STAT3 to α2-macroglobulin acute-phase response element, in the IL-6-mediated growth inhibition (and resistance to such inhibition), in human melanoma cell lines (unpublished observations).

Although a number of components have been identified in the IL-6 signalling pathway, most of their functions are related to stimulation of proliferation and the acute phase response. These may not be relevant for the present type of study, where growth inhibition is used as a criteria of biologic response. Therefore, analysis of cell cycle control is probably required. It is known that the cell cycle is controlled by many regulators, such as cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors, and that several genes or gene products are involved in its regulation, such as pRB, p53, TGFB, myc, fos, jun, etc. (10,157,181,383,454). It has been shown that TGFB can down-regulate c-myc RNA and protein, affect G1 cyclins, such as cyclin E, down-regulate CDK4 and up-regulate the CDK inhibitors, such as p15, or p27KIP1 (10,115,181). Studies on the response of early genes and that of cell cycle control machinery after exposure to IL-6 in human melanoma cells may provide important information on the mechanism of IL-6 growth inhibition and resistance to such inhibition. As discussed already, preliminary results by Dr. Floerenes have indicated an involvement of CDK inhibitors, such as p21WAF1 and p27KIP1 in IL-6 induced growth inhibition (115). Further investigation of the gene regulation of these cyclins, CDKs or CDK inhibitors in association with Stat3 tyrosine phosphorylation and DNA binding activity could
help to identify the changes or alterations in advanced-stage melanoma cells. Exogenous IL-6 might upregulate such universal CDK inhibitors in early stage melanoma cells, but fail to do so in their advanced-stage counterparts, through a Stat-dependent mechanism.

4. Assessment of other cytokines and growth factors in human melanoma cell lines in monolayer and non-monolayer cell culture:

In order to understand better the role that other cytokines and growth factors may play in regulating the growth and differentiation of human melanocytic cells, studies could be undertaken to test the activity of other such cytokines or growth factors, eg. IL-12 on normal, benign and malignant melanocytic cells. IL-12 was recently found to be able to suppress tumor growth in vivo by a paracrine mechanism (407), namely inhibition of angiogenesis (443). Others cytokines, such as leukoregulin and IL-4, can affect IL-8 and IL-6 gene expression, respectively, and may also affect the proliferation and differentiation of human melanocytic cells (50,272,341). In addition, analysis of the response to exposure of cytokines and growth factors in melanoma cells could be carried out in non-monolayer culture conditions. It has been shown that the response to growth factors or anticancer drugs observed in vitro monolayer cultures of tumor cells does not necessarily reflect the situation in vivo in solid tumors, or of the same cells grown under three dimensional multicellular spheroid conditions (222,238). Moreover, most cytokines can induce changes in extracellular matrix, and the extracellular matrix can in turn alter or affect the cellular response to cytokines (303). Therefore, experiments could be carried out to evaluate, or take into consideration, the context of cellular architecture and extracellular environments with respect to how paracrine growth modulators may affect the proliferation of melanoma cells obtained from different stages of disease progression.
5. Analysis of the responsiveness of melanoma cell lines obtained from different stage of progression to the treatment of chemotherapeutic drugs in comparison with their responsiveness to IL-6:

As discussed above, it has been shown that IL-6 can act as a survival factor for some types of malignant cell by inhibiting the process of apoptosis; moreover anti-IL-6 neutralizing antibodies can render tumor cells that express IL-6 more sensitive to the toxic effects of certain chemotherapeutic drugs in renal cell carcinoma and prostate carcinomas (48,282). Therefore, it is reasonable to speculate that the transition in growth responsiveness to IL-6 in the human melanoma cell lines we studied may be associated with some alterations in response to the treatment of anti-cancer drugs. It would not be surprising to find that the resistance to certain chemotherapy drugs may be associated with the resistant phenotype to growth inhibition, especially in relation to the autocrine production of IL-6 in melanoma cell lines.
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