Impact of intercellular adhesion on cell cycle and resistance to anticancer agents

by

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Abstract

Several studies have documented a correlation between cell-cell adhesion and resistance of tumor cells to anticancer agents. To assess directly the impact of intercellular adhesion on resistance of solid tumors, clonal variants of the mouse EMT/6 mammary carcinoma cell line were derived that formed tightly- or loosely-adherent aggregates in three-dimensional culture. As well, the enzyme hyaluronidase was found to disrupt aggregation of EMT/6 tightly-adherent variants. It was shown that tight intercellular adhesion, which correlated with reduced proliferation, was sufficient to confer resistance to anticancer agents which target rapidly dividing cells. Furthermore, the "anti-adhesive" hyaluronidase stimulated proliferation and sensitized tumor cells to anticancer agents both in vitro and in vivo in an ascites tumor model.

An analysis of the effect of intercellular adhesion on cell cycle regulators was conducted to examine the hypothesis that adhesion-dependent drug resistance may have a kinetic basis. These studies revealed that the cyclin-dependent kinase inhibitor p27Kip1 was upregulated by intercellular adhesion. Furthermore, depletion of p27 in EMT/6 cells increased cell proliferation and sensitized tumor cells to anticancer agents. These effects were observed in three-dimensional culture, but not in conventional monolayer culture.

E-cadherin is a homophilic cell-cell adhesion molecule found ubiquitously on normal epithelial cells. To determine if E-cadherin could mediate contact-dependent growth inhibition of non-adherent EMT/6 cells which lack endogenous E-cadherin, these cells were transfected with an...
exogenous E-cadherin expression vector. Aggregation of EMT/6-E-cadherin transfectants resulted in reduced growth and elevated p27 levels. Exposure to anti-E-cadherin neutralizing antibodies abrogated adhesion, stimulated growth, and reduced p27 levels. Further studies support the idea that E-cadherin-dependent growth suppression is mediated, at least in part, by elevated levels of p27.

The impact of cell adhesion on cancer has been studied almost exclusively in the context of tumor angiogenesis, invasion and metastasis. The studies reported here demonstrate that intercellular adhesion, through effects on p27, can also affect proliferation and resistance of solid tumors to anticancer agents. These studies suggest that tumor-targeted p27 antagonists or “anti-adhesives” may be useful chemosensitizers in conjunction with conventional anti-cancer therapy.
Acknowledgements

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Chapter 1

General Introduction
1.1 The Problem of Drug Resistance

Cancer is the second leading cause of mortality in North America, and at least one out of three individuals will develop cancer during their lifetime, and one out of five will die of the disease (Li and Kantor, 1997). Surgery was the first effective treatment for cancer and its success rate has gradually increased over the course of the century with the development of better diagnostic and surgical equipment, and more refined techniques. It is still the most widely used method for treating cancer, and accounts for the largest number of cures. However, in many cases surgery is unsuccessful and the disease either returns at the primary tumor site or, more commonly, it metastasizes to distant locations. For certain types of cancer, surgical removal of the primary tumor is difficult or impossible, due to the location of the tumor (e.g. brain tumors) or because the cancer is disseminated from the outset of the disease (e.g. leukemias). In these situations, chemotherapy or radiotherapy become the front-line arsenal in treatment of the disease. Chemotherapy is most frequently used for disseminated disease whereas radiotherapy is used when the tumor is localized to one region. Unfortunately, many tumors, especially the more common solid tumors such as those of the breast, colon and lung, often fail to respond to such forms of therapy. Thus, resistance to chemo- or radiotherapy is considered to be the most significant impediment to the cure of neoplastic disease (Scotto and Bertino, 1997).

The development of resistance, or acquired resistance, occurs in 30-40% of cancer patients. Tumors are considered intrinsically or de novo resistant if they fail to respond from the onset of cytotoxic therapy and this occurs in approximately 60% of cases. However, not all tumors fail such treatment. A minority of tumors are curable by chemotherapy or radiation therapy and these include testicular carcinoma, acute lymphocytic leukemia, Hodgkin's disease, diffuse histocytic lymphoma,
Burkitts lymphoma, and choriocarcinoma (Sobrero and Bertino, 1986; Tannock, 1994). Although these tumor types are rare, much optimism has arisen from their successful eradication because they provide a strong argument that the more common types of cancers, such as those of the breast, colon, lung and prostate can also be eradicated provided enough therapy can be administered to ensure that every malignant cell is killed. Such cures are important to bear in mind when considering the fact that tumor cells are genetically unstable and seem to have a formidable ability to develop resistance against any type of cytotoxic anticancer therapy. Unfortunately, the extent of tumor cell kill is seldom sufficient to eradicate every malignant tumor cell as the amount of therapy necessary would, in most cases, be unacceptably toxic to the patient. Bone marrow cells or gut mucosal cells are critical host tissues that, due to their inherent sensitivity, typically limit the amount of chemo- or radiation therapy that can be administered. The therapeutic index is a measure of the relative cytotoxicity of therapy against tumors as compared to normal tissue. Depending on several factors, such as patient age, the tumor type, the preferred treatment regimen, etc., oncologists are often faced with a small or absent therapeutic index making it virtually impossible to achieve a cure. Hence, a major goal of many research laboratories including our own, is to devise ways to increase the therapeutic index, which in theory should lead to an increase in survival rates.

Three general approaches have been taken in order to increase the therapeutic index. The first involves localizing therapy to the target of interest. Radiotherapy is the most successful and widely used method for regional treatment of disease, and is especially useful against solitary inoperable tumors. Intraperitoneal chemotherapy for the treatment of advanced ovarian carcinoma is also proving effective, and its efficacy is based on an increased peritoneal fluid to plasma drug ratio (Markman, 1996; Schneider, 1994). Although the peritoneal membrane is permeable, slow
systemic uptake of the drug and rapid subsequent drug clearance from the plasma both help to increase local drug concentrations. The rate of diffusion is inversely proportional to the square root of the molecular weight of the drug. Thus, high molecular weight drugs, such as Taxol, are retained in the peritoneal cavity longer than small molecular weight drugs such as cisplatin. Once drugs are absorbed through the peritoneal cavity they enter capillaries of the portal circulation and pass through the liver. Hence, drugs which are inactivated in the liver also display favourable peritoneal fluid to plasma drug ratios following intraperitoneal administration. Because Taxol also falls into this category, it may prove to be a drug of choice for future intraperitoneal chemotherapy (Markman et al., 1995). Phase I clinical studies have demonstrated that Taxol injected intraperitoneally results in 1000 times higher drug concentrations in the peritoneal cavity than in the systemic compartment (Markman et al., 1992) (see Table 1.1). Although cisplatin injected intraperitoneally displays less favourable pharmacokinetics, recent clinical studies have demonstrated a significant prolongation of patient survival when compared with standard intravenous administration (Alberts et al., 1996).

Ironically, a major limitation with intraperitoneal therapy, especially for high molecular weight drugs, is that drug penetration through large vascularized tumor nodules may be more difficult to achieve than if the drug was administered intravenously (Dedrick and Flessner, 1997). Indeed, intraperitoneal therapy has proven most effective in cases where residual intraperitoneal tumor nodules are of a small size (i.e. <2 cm in greatest dimension) (Alberts et al., 1996; Kirmani et al., 1991).

The second method used to enhance the therapeutic index involves increasing the resistance of normal host tissues to therapy. For example, in cases where bone marrow toxicity is limiting, cytokines have been used to enhance the recovery of bone marrow cells following anticancer therapy.
Table 1.1 Pharmacokinetic Advantage Associated with Intraperitoneal Administration of Selected Cytotoxic Agents Active in Ovarian Cancer

<table>
<thead>
<tr>
<th>Agent</th>
<th>Peak peritoneal cavity to plasma concentration ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>20</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>18</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>474</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>620</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>71</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>298</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1000</td>
</tr>
</tbody>
</table>

(Nemunaitis, 1997; Neidhart 1993). Higher doses of therapy can also be achieved by giving an autologous bone marrow transplant following anticancer treatment. Such strategies have enabled the use of high-dose chemotherapy that, in the absence of bone marrow support, would almost certainly be lethal to the patient. Indeed, for metastatic breast cancer high-dose therapy results in higher initial response rates even in tumors that were previously non-responsive. Furthermore, recent phase II clinical trials suggest that high-dose treatment may increase 3 year survival rates by 10-20% (reviewed by Girouard and Crump, 1996). Results from such studies are encouraging in that they suggest that drug resistance was partially overcome, at least initially, and that the therapeutic window was enhanced. Nevertheless, this type of treatment is also hindered by many factors, including among others, tumor cell contamination of bone marrow support and unacceptable levels of toxicity in a subgroup of patients. Furthermore, the evidence that high-dose chemotherapy with bone marrow support is superior to conventional therapy in treating metastatic breast cancer is still preliminary, and convincing results from large randomized clinical trials are only beginning to emerge (Girouard and Crump, 1996; Crown, 1997).

The third, and perhaps the most commonly attempted method for enhancing the therapeutic window involves increasing the sensitivity of tumor cells to therapy. Arguably, this approach has been less successful to date and clinical studies employing the use of chemo- or radiation sensitizers in combination with anticancer therapy have been mostly disappointing (Houghton and Kaye, 1994). Nevertheless, the approach has a strong conceptual basis, and has only one target (ie. the tumor). Historically, a major obstacle limiting the utility of this approach has been lack of specificity for tumor cells. Lack of a true understanding of the reasons why tumors in patients resist anticancer therapy has also limited this approach. A major goal of the research program in our laboratory has
been to uncover the cellular and molecular mechanisms underlying clinical drug resistance.

1.1.1 In Vitro Versus In Vivo Drug Resistance Models

The vast majority of research on biochemical mechanisms underlying drug resistance have employed drug-resistant cell lines selected in vitro using a single experimental approach. The method used involves exposing cells growing in conventional monolayer tissue culture to a continuous or stepwise addition of increasing concentrations of drug. This approach has led to the discovery of many biochemical drug resistance mechanisms including, reduced drug accumulation, increased drug efflux, increased metabolic drug detoxification, and increased DNA repair (Scotto and Bertino, 1997). Since its discovery, P-glycoprotein has been one of the most intensively studied mechanisms of drug resistance. This cell surface drug efflux pump is responsible for conferring a multidrug resistance phenotype (to natural products) on drug-selected cell lines in vitro. Undoubtedly, the discovery of such resistance mechanisms was facilitated by the high levels of drug-resistance (often several hundred fold) obtainable in cell lines selected for drug resistance in monolayer tissue culture. However, it is still unclear what impact, if any, such mechanisms have on acquired clinical drug resistance which is often on the order of 3-4 fold. Furthermore, attempts to develop more effective treatment strategies based on knowledge of such drug resistance mechanisms have frequently failed to live up to expectations, especially for solid tumors. (Sobrero and Bertino, 1986; Houghton and Kaye, 1994).

By use of cell lines selected for drug resistance in vivo instead of in tissue culture, it may be possible to uncover resistance mechanisms that have a greater probability of explaining the basis of clinical drug resistance in solid tumors. However, such in vivo-derived drug-resistant cell lines are
rare, and based on a thorough search of the literature over the past 5 years, less than 20 laboratories worldwide have ever employed their use for studying drug resistance of solid tumors (see table 1). In sharp contrast, hundreds of papers are published annually describing the use of drug-resistant variants derived by the conventional method of drug-selection in monolayer culture. This surprising paucity of reports may be partly explained by the fact that in vivo drug-selection is more labour intensive, costly, and often more time consuming than in vitro drug-selection. However, in some cases such models can reveal resistance mechanisms that would otherwise be undetectable in conventional monolayer tissue culture. For example, in 1991 Dr. Beverly Teicher and co-workers derived alkylating agent-resistant variants of the EMT-6 mouse mammary carcinoma by repeated drug exposure of syngeneic tumor bearing BALB/c mice. Although highly resistant in vivo, when the subcutaneous tumors were removed and adapted to monolayer tissue culture, the drug-selected variants unexpectedly lost their resistance phenotype. The drug resistance phenotype was stable, however, because reinoculation of the cultured cells into nude mice resulted in remanifestation of the drug resistance profiles. In another study, a human lung adenocarcinoma cell line grown subcutaneously in nude mice was made resistant by repeated exposure to a vinca alkaloid-monoclonal antibody conjugate or to the unconjugated drug. Again resistance was only detected in vivo but not in monolayer culture (Starling et al., 1990).

### 1.1.2 The multicellular spheroid model

Intrigued by Dr. Teicher’s studies, a postdoctoral fellow from our laboratory, Dr. Hiroaki Kobayashi, began a series of experiments designed to test whether or not the drug resistance profiles of EMT-6 drug resistant variants observed in vivo could be recapitulated in vitro. The rationale for
Table 1.2 Solid tumor models selected for drug resistance in vivo.

<table>
<thead>
<tr>
<th>Parent cell line</th>
<th>Drug(s) used for in vivo selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMT-6 mouse mammary carcinoma</td>
<td>cyclophosphamide, cisplatin, carboplatin, thiotepa</td>
<td>Teicher <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>UCLA-P3 human lung adenocarcinoma</td>
<td>mAb-vinca alkaloid immunonoconjugates</td>
<td>Starling <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>A2780 human ovarian carcinoma</td>
<td>cisplatin</td>
<td>Rose and Basler, 1990</td>
</tr>
<tr>
<td>O-342 rat ovarian tumor</td>
<td>cisplatin</td>
<td>Chen and Zeller, 1990</td>
</tr>
<tr>
<td>TE-671 human rhabdomyosarcoma</td>
<td>melphalan</td>
<td>Rosenberg <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>T4-O1320 mouse mammary tumor</td>
<td>cyclophosphamide</td>
<td>Takeda <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>HxRh12 and HxRh28 human rhabdomyosarcoma</td>
<td>vincristine, L-PAM</td>
<td>Horton <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>PEO1 and PEO4 human ovarian adehocarcinomas</td>
<td>cisplatin, chlorambucil, 5-flourouricil</td>
<td>Wolf <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>Lewis lung carcinoma</td>
<td>MeCCNU</td>
<td>Stephens <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>MT mouse mammary carcinoma</td>
<td>melphalan, cisplatin, cyclophosphamide</td>
<td>McMillan <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>HX78 human small cell lung cancer</td>
<td>cyclophosphamide</td>
<td>Berman and Steel, 1984</td>
</tr>
<tr>
<td>M5076 ovarian reticular cell sarcoma</td>
<td>cyclophosphamide</td>
<td>D’Incalci <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>RD human rhabdomyosarcoma</td>
<td>vincristine</td>
<td>Houghton <em>et al.</em>, 1981</td>
</tr>
<tr>
<td>HX series of human bronchial carcinomas</td>
<td>cyclophosphamide, vincristine, cyclophosphamide, CCNU methotrexate, 5-flourouricil Adriamycin, bleomycin,</td>
<td>Shorthouse <em>et al.</em>, 1980</td>
</tr>
</tbody>
</table>
this approach was that it could simplify the process of uncovering the mechanism responsible for the drug resistance, provided that the mechanism was not host cell-dependent. Initial experiments, which involved growing cells in vitro on various ECM components such as fibronectin, collagen and matrigel, failed to rescue the drug resistance phenotype. Subsequently, however, it was found that the drug resistance properties of these cells could indeed be revealed in vitro if the cells were grown in three-dimensional culture (i.e., as multicellular tumor spheroids) (Kobayashi et al., 1993). This resistance was referred to as "acquired multicellular resistance". Drug resistance in three-dimensional culture was also associated with a striking increase in cell-cell adhesion (or compaction) of the drug resistant variants compared with the loosely-adherent EMT-6 parental cell line (EMT-6/P). A subsequent study demonstrated, using both the mouse EMT-6/P cell line and the human MDA-MB-231 breast cancer cell lines, that a transient increase in both aggregate compaction and drug resistance can occur rapidly following exposure to 4-hydroperoxycyclophosphamide (4-HC), an activated form of cyclophosphamide (Graham et al., 1994). Together, these studies suggested the possibility that intercellular interactions may be necessary for the manifestation of certain forms of acquired drug resistance in cancer (Kerbel et al., 1994).

1.2 Cell Adhesion

1.2.1 Cell Adhesion and Cell Survival

It was first realized less than 5 years ago that cell-cell or cell-matrix adhesion can regulate apoptosis or programmed cell death (Meredith et al., 1993; Frish and Francis, 1994; Bates et al., 1994, Montgomery et al., 1994; Brooks et al., 1994). Since then, numerous papers have confirmed and extended this finding to include many endothelial and epithelial cell types (Rak et al., 1995;
Hermiston and Gordon, 1995a; Howlett et al., 1995; Zhang Z. et al., 1995; Boudreau et al., 1995; Boudreau et al., 1996; Strater et al., 1996; Peluso et al., 1996; Hague et al., 1997; Aoshiba et al., 1997; Rozzo et al., 1997). Normal cells appear to rely heavily on interactions with the extracellular matrix for their survival, and these interactions are mediated by integrins, most often involving the $\beta_1$-integrin or $\alpha_v$-integrin subunit (see section on 'Cell Adhesion Molecules') (Meredith et al., 1993; Brooks et al., 1994; Montgomery et al., 1994; Bates et al., 1994; Howlett et al., 1995; Zhang et al., 1995; Strater et al., 1996; Rozzo et al., 1997; Aoshiba et al., 1997). Early during tumor progression, tumor cells appear to lose their dependence on cell-matrix interactions, but maintain their requirement for cell-cell interactions (Hague et al., 1997). Later in tumor progression, cell-cell interactions often become weaker, and this may aid tumor cell dissemination from the primary tumor site. However, late stage tumors maintain some level of intercellular adhesion, or the ability to reactivate adhesion suggesting that loss or gain of cell-cell adhesion in tumors may be a dynamic process (Kobayashi et al., 1993; St.Croix et al., 1996a; Bracke et al., 1994). An increased level of adhesion is thought to facilitate survival of tumor emboli and, as our studies suggest, may also help tumor cells to evade the cytotoxic effects of anticancer therapy (Kobayashi et al., 1993; St. Croix et al., 1996a). Recent studies by Dr. Rakesh Jain and co-workers demonstrate that the continuous growth of solid tumor cells in a confined space with external pressure results in a high 'packing density', solid stress and increased resistance to apoptosis (Helmlinger et al., 1997). Such external pressure may prevent apoptosis by promoting tighter intercellular interactions. Thus, during the various stages of tumor progression, adhesion molecules may function as part of a survival mechanism. Because apoptosis can be induced by most, if not all, anticancer agents, we postulated that cell-cell or cell-ECM interactions may protect cells from apoptosis induced by anticancer agents
(St.Croix et al., 1996a). Indeed, experimental support for this idea has recently been reported (Whitacre and Berger, 1997).

Although it is unclear exactly how cell-cell or cell-ECM interactions protect cells from undergoing apoptosis, some components involved in the regulation of this pathway have recently been uncovered. Most studies have analyzed the effects of cell-ECM interactions on apoptosis of normal epithelial cells in culture. Apoptosis induced by detachment of normal cells from their underlying ECM has been termed "anoikis" which is Greek for 'homelessness' (Frisch and Francis, 1994). The proto-oncogene bcl-2 was the first molecule to be analyzed in this context because of its known ability to protect cells from apoptosis induced by various other physiological stimuli (Frisch and Francis, 1994). Frisch and Francis demonstrated that overexpression of bcl-2 protects MDCK normal kidney cells from anoikis. Subsequently, several other reports have supported a possible role for bcl-2, as well as other related family members such as bax, in regulating anoikis (Zhang Z. et al., 1995; Frish et al., 1996; Merio et al., 1997; Aoshiba et al., 1997).

A family of proteases known as caspases have also been implicated in signaling pathways regulating apoptosis (Fraser and Evan, 1996). During anoikis caspases have been shown to be active, and inhibitors of these proteases prevent anoikis (Cardone et al., 1997; Boudreau et al., 1995; Frisch et al., 1996). Recently, it was shown that caspases cleave and activate MEKK during anoikis (Cardone et al., 1997). This may be a crucial step because transfection of a dominant negative MEKK which is unaffected by caspases suppresses anoikis while wild-type MEKK promotes apoptosis. Overexpression of bcl-2 was shown to suppress caspase activity suggesting that caspases and MEKK activation are downstream of bcl-2 (Frisch et al., 1996).

Members of the Stress Activated Protein Kinase (SAPK) family, also known as Jun-N-
terminal Kinases (JNK), have also been implicated in apoptosis and are activated in response to many apoptotic stimuli, including TNF-α, anisomycin, sodium arsenite, UV irradiation, as well as anticancer agents such as cisplatin (Woodgett et al., 1996; Zanke et al., 1996). Frisch and co-workers have shown that SAPK is activated when cells are detached from their underlying ECM. (Frisch et al., 1996). Importantly, blocking the SAPK pathway using dominant negative mutants protected cells from anoikis (Frisch et al., 1996). Because SAPK is known to be activated by MEKK, detachment of normal cells from ECM may promote anoikis through a pathway involving integrins, bcl-2, caspases, MEKK and SAPK.

While the molecular mechanisms regulating anoikis are only beginning to be elucidated, even less is known about how cell-cell interactions protect cells from apoptosis. One recent study reported that bcl-2 was overexpressed in bronchial epithelial cells upon cell-cell aggregation (Aoshiba et al., 1997). Interestingly, anisomycin and arsenite which are two of the most potent inducers of SAPK in monolayer culture (Woodgett et al., 1996), failed to activate SAPK of EMT-6 cells grown as spheroids in three-dimensional culture (Dr. B. Zanke, personal communication). Hence, cell-cell interactions may prevent the activation of apoptotic pathways involving bcl-2 and/or SAPK, but further investigation into this area is needed.

1.2.2 Cell Adhesion and Drug Resistance

It is well known that tumor spheroids express what might be called “multimodality resistance”, that is, they not only express intrinsic resistance to a diverse class of cytotoxic chemotherapeutic drugs, but also to such agents as ionizing radiation (Sutherland, 1988; Olive and Durand, 1994), immunotoxins (Chignola et al., 1995), activated killer cells (Ochalek and Kleist,
1994), complement mediated cell lysis (Bjorge et al., 1997), cytostatic cytokines (Gorlach et al., 1994) and hyperthermia (Sutherland, 1988; Olive and Durand, 1994). The mechanisms underlying this pleiotropic resistance are probably equally diverse and may include microenvironmental influences, reduced penetration of the therapeutic agent (Ochalek and Kleist, 1994) and resistance to apoptosis (Helmlinger et al., 1997). For example, several studies have demonstrated that high molecular weight drugs such as Adriamycin penetrate through tumor spheroids poorly (for review see Sutherland, 1988). Because spheroids are most often considered relatively resistant based on a comparison with the same cells grown in monolayer culture, intercellular adhesion may be indirectly involved in all such forms of resistance.

Twenty-five years ago Sutherland and Durand reported that cell-cell contact may directly affect resistance of tumor cells to anticancer agents (Durand and Sutherland, 1972; Sutherland, 1988; Olive and Durand, 1994). Their conclusions were based on the fact that small multicellular tumor spheroids were highly resistant to anticancer agents compared to monolayer cultures of the same cells, and this resistance could not be explained solely on the basis of microenvironmental influences or reduced drug penetration into the spheroid core. This led them to postulate that a cell-cell “contact effect” was responsible for resistance; however, the molecular mechanisms governing the contact effect still remain unclear (Olive and Durand, 1994). Likewise, cells grown as aggregates in collagen gels also show elevated levels of resistance to many cytotoxic agents compared to monolayer cultures, which again could not be attributed to decreased drug penetration (Miller et al., 1985; Hoffman, 1993; Hoffman, 1994). Dimanche-Boitrel and co-workers have been studying what may be a similar phenomenon, that is, drug resistance that develops as cells reach confluence in monolayer culture (Dimanche-Boitrel et al., 1992; Dimanche-Boitrel et al., 1993). Once again, this
resistance, which they refer to as "confluence-dependent resistance", could not be explained solely on the basis of known drug resistance mechanisms, such as reduced drug accumulation or increased levels of P-glycoprotein.

Although the precise mechanism(s) underlying these forms of resistance remain unclear, cells grown either to confluence in monolayer cultures or as aggregates in three-dimensional cultures, like solid tumors in vivo, almost always display a much lower growth fraction than subconfluent monolayer cultures. Thus, it has been postulated that an adhesion-dependent reduction in tumor cell growth may be essential for the development of this type of resistance and may protect cells from drug-induced apoptosis (Dimanche-Boitrel et al., 1993; St.Croix et al., 1996a). Conversely, rapidly dividing cells may be more sensitive to undergoing apoptosis (see below). In that regard, Garrido et al. have recently shown that the heat shock protein hsp27, which is induced by hyperthermia and can prevent cells from undergoing drug-induced apoptosis (Samali and Cotter, 1996), is upregulated by cell-cell contact (Garrido et al., 1997). Because forced overexpression of hsp27 in subconfluent cultures protects cells from apoptosis, it has been proposed that hsp27 may be involved in confluence-dependent resistance of tumor cells (Garrido et al., 1997).

The aforementioned studies were primarily conducted on cell lines that had never been pre-exposed to drugs and thus may help to explain the intrinsic drug resistance observed in many solid tumors growing in vivo. Multicellular drug resistance resembles, at least superficially, the contact effect or confluence-dependent resistance. However, initial studies on multicellular drug resistance suggested that this form of resistance may contribute not only to intrinsic drug resistance of tumors, but also to acquired resistance (Teicher et al., 1991, Kobayashi et al., 1993). Regardless of whether resistance is acquired or intrinsic, increased cell-cell adhesion may be necessary for resistance in
each of these cases, and these forms of resistance may share a molecular basis.

1.2.3 Cell Adhesion Molecules

The aforementioned studies suggested a role for cell adhesion in regulating resistance to anticancer agents. However, the exact adhesion mechanism regulating adhesion in EMT-6 cells remains elusive although hyaluronic acid (HA) appears to be involved (see chapter 2). Briefly summarized in this section are details about the five major families of cell-cell and cell-substratum adhesion receptors including integrins, members of the immunoglobulin superfamily, selectins, cadherins and CD44 (see figure 1.1). More emphasis is placed on the CD44 family of adhesion molecules because of their ability to bind HA. The cadherin family is also highlighted because of data we have recently obtained demonstrating that E-cadherin can regulate both cell adhesion and proliferation of tumor cells (see chapter 4).

**Integrins** are obligate heterodimers composed of both an α and β-subunit. There are 16 known α chains and nine known β-chains which can combine in various combinations to form at least 24 different heterodimers (Gille and Swerlick, 1996). Integrin receptors have been found on all cell types, and most bind to ECM components such as collagen, laminin and fibronectin. However, some integrins, particularly those found on leukocytes, can bind to cell adhesion molecules (CAMs) of the immunoglobulin superfamily (i.e. heterophilic adhesion) and can mediate binding to different cell types (i.e. heterotypic adhesion). Divalent cations (Ca\(^{2+}\), Mg\(^{2+}\) or Mn\(^{2+}\)) are required for the adhesive interactions of integrins (Hynes and Lander, 1992).

Members of the immunoglobulin superfamily comprise another major class of cell adhesion receptors. Neural cell adhesion molecule (NCAM) was the first to be characterized, but it is now
Figure 1.1 Major Families of Cell Adhesion Receptors. Cadherins are Ca\(^{2+}\)-dependent homophilic cell-cell adhesion molecules. Immunoglobulin superfamily adhesion receptors contain immunoglobulin domains (Ω) and, frequently, fibronectin type III repeats (striped boxes). Selectins contain a Ca\(^{2+}\)-dependent C-type lectin domain, a single EGF-like repeat (stippled box), and a series of repeats (ovals) related to those of complement-binding proteins. Integrins are divalent cation (M\(^{+}\)) binding heterodimeric receptors. The CD44 receptor, represents the "hematopoietic" or "standard" form which binds HA. •, indicates potential N-linked glycosylation sites; ○, potential O-linked glycosylation sites; =>, potential sites for addition of condroitin sulfate; ←, site of insertion of alternatively spliced exons. Binding domains are indicated by the darkest colorings. [Adapted from (Hynes and Lander, 1992) and (Lesley et al., 1993)].
clear that many members of this large and diverse family function as adhesion receptors [for example, intercellular adhesion molecules (ICAM-1 and ICAM-2), vascular cell adhesion molecule (VCAM) and platelet-endothelial cell adhesion molecule (PECAM-1)]. All immunoglobulin superfamily members are divalent cation-independent although some, for example NCAM, display homophilic adhesion whereas others, for example ICAM-1 and ICAM-2 (which bind integrins) adhere in a heterophilic manner. These receptors typically contain several immunoglobulin-related domains and fibronectin type III repeats. Some of them are transmembrane as depicted in figure 1, but others, such as CEA, are linked to the cell surface via a glycosylphosphatidyl inositol tail (Buck, 1992; Hynes and Lander, 1992).

*Selectins* are carbohydrate binding proteins found on blood cells and endothelial cells. Selectins contain a single calcium binding site in their C-type lectin domain, responsible for binding specific carbohydrate groups. They also share sequence similarity to EGF and complement binding proteins. Three selectins have been well characterized including L(leukocyte)-selectin, P(platelet)-selectin and E(endothelial)-selectin. Selectins mediate the initial attachment of leukocytes to endothelial cells lining blood venules (Hynes and Lander, 1992).

The *CD44* family of cell surface glycoproteins mediate divalent cation-independent binding to HA, a major component of the ECM made up of repeating units of the disaccharide β-1,4-glucuronic acid-β-1,3-N-acetylglucosamine. Binding to HA is mediated by the N-terminal region of CD44 which contains a disulfide bond-stabilized loop structure (Lesley *et al.*, 1993). Attachment of O- or N-linked carbohydrate side chains has been shown to affect the affinity of CD44 for HA (Bartolazzi *et al.*, 1996; Katoh *et al.*, 1995). CD44 is also involved in cell migration, lymphopoiesis and lymphocyte homing. Although CD44 is encoded by a single gene, this family is very diverse.
due to alternative splicing which results in at least 20 different isoforms (Naot et al., 1997). A number of variant forms have been isolated in certain cell types and in some cases may promote the formation of metastasis (Gunthert et al., 1995). However, in most cell types two major forms of CD44 predominate; the 85-95 Kd CD44H (CD44S) hematopoietic or "standard" form and the 180-200 Kd CD44E "epithelial" form. Although the binding of CD44 to HA is well established there is mounting experimental evidence that CD44 may also bind weakly to other components of the ECM such as fibronectin, collagen, and laminin (Jalkanen et al., 1992; Ishii et al., 1993). Importantly, adhesion mediated by CD44 is considered to be relatively weak in comparison to other adhesion mechanisms such as those involving integrins or cadherins. (Underhill, 1992).

Cadherins are a large family of calcium-dependent integral membrane proteins that are found in virtually every type of multicellular tissue. The family is comprised of at least 20 different members and can be subdivided into six different gene families (Aberle et al., 1996). Type I classical cadherins have been the most extensively studied and include N-cadherin (neural), P-cadherin (placental) and E-cadherin (epithelial). Classical cadherins contain 5 tandem repeats in their extracellular domain, the most distal domain containing the binding site responsible for homophilic binding between apposing cadherin molecules on adjacent cells (Aberle et al., 1996). Although the binding of monomeric cadherins is thought to be weak, structural analysis suggests that the binding of multiple cadherin molecules leads to the formation of a strong zipper-like superstructure (Gumbiner et al., 1996). Between the five cadherin repeats lie four calcium binding sites which are necessary for cadherin-mediated adhesion. By stabilizing the binding of successive repeat domains, calcium binding ensures that the cadherin projects from the cell surface and forms a rod-like structure (Aberle et al., 1996).
The amino acid sequence of classical cadherins varies most extensively in the extracellular region while the cytoplasmic domain is the highly conserved. The variation in the extracellular region is thought to confer cell type specificity, since cell mixing experiments have shown that cells can segregate according to the type of cadherin expressed at the cell surface (Nose et al., 1988). The conserved intracellular region serves as an attachment site for catenins, cytoplasmic proteins which link all classical cadherins to the underlying actin cytoskeleton (for diagram see figure 5.3, Chapter 5). β-catenin and γ-catenin (also called plakoglobin) can bind directly to same region of the cadherin intracellular tail. Both β- and γ-catenin can bind to α-catenin, and α-catenin is essential for linking cadherins to the actin cytoskeleton. Interestingly, immunoprecipitation studies suggest that β-catenin and γ-catenin form mutually exclusive complexes between cadherin and α-catenin (Nathke et al., 1994). Other proteins such as p120CAS have also been found in cadherin-catenin complexes. However, the role of such molecules in adhesion or signaling (see section 1.3.5) is currently unclear (Reynolds et al., 1994; Shibamoto et al., 1994).

1.3 Cell Cycle

1.3.1 The Impact of Growth Kinetics on Drug Resistance of Spheroids and Solid Tumors

For years it has been known that multicellular spheroids, like solid tumors, typically have a low growth fraction. For example, Durand has shown using tightly adherent spheroids of V79 Chinese hamster lung cells that the growth fraction of spheroids decreases dramatically with spheroid age and size (Durand 1990). In these studies, the growth fraction decreased from 100% in monolayers to 35% in spheroids, while the cell cycle time was only slightly elongated. Numerous other studies comparing the kinetics of monolayers with tumor xenographs and spheroids have found
similar results (Yuhas et al., 1977; Yuhas et al., 1978; Freyer and Sutherland, 1980; for review see Durand 1990). The effect of packing density on growth kinetics in three-dimensional culture was not analysed in any of these studies. It is noteworthy, however, that solid tumors typically decrease their growth as they enlarge (Steel, 1977). Based on recent studies by Dr. Rakesh Jain and co-workers the external solid stress surrounding a tumor in a confined tissue compartment may force an increased packing density and tighter intercellular adhesion as the tumor grows. In turn, tighter intercellular adhesion may result in a decrease in tumor growth (Helmlinger et al., 1997).

Not only do three-dimensional tumor cell cultures have a low growth fraction, they also, like solid tumors, display resistance to a wide variety of cytotoxic agents compared with monolayer cultures (Sutherland 1988; Hoffman 1991; Olive and Durand, 1994). Given the preferential activity of most currently used anticancer agents towards rapidly dividing cells (Tannock, 1978; Tannock, 1994), it follows that tumor growth fraction is likely to be a critical factor limiting tumor responsiveness to therapy. Most currently used anticancer agents preferentially target rapidly proliferating cells for at least two general reasons. First, many chemotherapeutic drugs were originally designed to target dividing cells (e.g. methotrexate and 5-flourouracil). Such drug design was based on the presumption that the most significant difference between tumor cells and normal cells was their growth capacity, an assumption that in hindsight is not always true, especially for the more common solid tumors. Second, in vivo models previously employed for drug screening have relied heavily on rapidly growing mouse leukemia cell lines for identifying active chemotherapeutic agents (Grindey, 1990; Khleif and Curt, 1997). Although cells from common solid tumors of human origin were introduced into such screens in the mid-1980's, for cost-effectiveness only compounds found to be active in first-pass screens against rapidly growing mouse leukemias were evaluated on
such solid tumors. Currently, first-pass drug screens generally employ human tumor cell lines from monolayer tissue culture (Khleif and Curt, 1997). Although such screens are beneficial in terms of cost and labor, the disadvantages are obvious as the growth fraction of such cultures are likely to be artificially high. Thus, it is not surprising that many chemotherapeutics, including cyclophosphamide, are known to preferentially kill cells in rapidly growing tumors even though the mechanisms by which they elicit this death remain obscure.

Given the preferential activity of most chemotherapeutics towards dividing cells, it is understandable why the few cancers that are curable by chemotherapy, such as childhood tumors, testicular carcinoma, and some leukaemias and lymphomas tend to be rapidly growing and have high growth fractions (~30%) (Tannock, 1994; Grindey, 1990). Likewise breast tumors with a high S-phase fraction almost always respond better, at least initially, than those with a low S-phase fraction (Amadori et al., 1997; Heitanen, 1995) although effects on survival have been variable. In contrast, common solid tumors which typically do not respond to chemotherapy, such as colon, prostrate, and some breast cancers often have very low growth fractions ranging between 1-15% (Tannock, 1978; Tannock, 1994; Meyer, 1982).

Although the molecular mechanisms governing the preferential activity of cytotoxic agents towards rapidly growing tumors are, generally, still obscure, it is likely that proliferating cells are more susceptible to apoptosis. This is supported by empirical data, as well as recent molecular studies demonstrating that key regulatory molecules such as p21, p27, pRb, p53, bcl-2, and myc influence both cell cycle and apoptosis in a co-ordinated manner (Poluha et al., 1996; Wang and Walsh, 1996, Waldman et al., 1996; St. Croix et al., 1996b; Haas-Kogan et al., 1995; Smith and Fornace, 1996; Borner, 1996; Amati et al., 1993; Oren, 1992; Evan et al., 1992; Askew et al., 1991).
Thus, growth promoting molecules such as myc also promote apoptosis, and molecules such as bcl-2 which prevent apoptosis arrest cells in G1 (Amati et al., 1993; Evan et al., 1992; Askew et al., 1991; Borner, 1996; Linette et al., 1996). The latter effect of bcl-2 may be a result of its ability to upregulate p27 protein levels (Linette et al., 1996). Such results clearly demonstrate the intimate connections between apoptosis and proliferation and suggest the existence of complex autoregulatory feedback loops regulating both processes. One may ask, if growth and death are so equally balanced, how then do tumors grow? Perhaps the most likely explanation is that tumor cells only need proliferate slightly faster than they die in order for the population to expand. Indeed, for normal adult tissues to maintain homeostasis, a precise balance between cell birth and cell death is essential. Thus, certain oncogenic changes, in addition to stimulating growth may, in some cases, simultaneously activate an increased base level of apoptosis as part of an automatic compensatory feedback loop. If an increase in the base level of apoptosis sensitizes tumor cells to undergoing further drug-induced apoptosis, then this could help to explain the increased sensitivity of proliferating tumor cells to cytotoxic therapy. Likewise, normal tissues which proliferate rapidly would also be expected to have a higher base level of apoptosis, which could also help to explain their chemosensitivity. For all the aforementioned reasons, adhesion-dependent growth suppression is likely to be useful in understanding drug resistance of solid tumors. Recent advances made in the understanding of the molecular regulation of cell cycle progression provides a valuable new avenue for delineating the mechanisms underlying contact-dependent growth inhibition.

1.3.2 The Cell Cycle

The mammalian cell cycle can be divided into four broadly defined phases: the G1 phase
(Gap1) when the cell prepares to replicate its DNA, the S phase (Synthesis) when DNA synthesis and replication takes place, the G2 phase (Gap2), when the cell prepares to divide, and M phase (Mitosis) during which time cell division takes place (see figure 1.2). For normal cell division to occur a conserved family of protein kinases, known as cyclin-dependent kinases (cdks), have to be activated and inactivated in a very precise and sequential manner. Cdk are activated by binding to small molecules called cyclins, the levels of which fluctuate during the cell cycle. Activity of cyclin/cdk complexes is also regulated by specific phosphorylation and dephosphorylation of the kinase or by direct binding of cdk inhibitory molecules (Morgan, 1995).

Four major cyclin-dependent kinases are widely expressed and are required for normal cell cycle progression. Cdk4 and cdk6 are active during the G1 phase of the cell cycle, cdk2 during the G1 to S phase transition, and cdc2 (cdk1) during the G2/M phase. D-type cyclins (cyclins D1, D2 and D3) interact with and activate cdk4 and cdk6 during the G1 phase of the cell cycle. The particular D-type cyclin expressed may depend on the cell type, although cell lines often express more than one D-type cyclin. Late in G1, cyclin E associates with and activates cdk2, and this is thought to control entry into S phase. Upon entry into S phase, cyclin E is degraded and replaced by cyclin A which associates with cdk2 and is required for DNA synthesis. Finally, cdc2 complexed with cyclin B regulates entry into mitosis (Morgan, 1995).

Although cdk are thought to play a critical role in cell cycle progression, the substrates for these kinases remain relatively uncharacterised. The retinoblastoma tumor suppressor protein (pRb) is thought to be the major physiological substrate for the D-type cyclins complexed with cdk4 or cdk6, but it may also be a substrate of cyclinE/cdk2. Three Rb family members are currently known; pRb, p130 and p107. Proteins of the Rb family bind to and inhibit activity of members of the E2F
Figure 1.2 The Cell Cycle
family of transcription factors. Phosphorylation of pRb by cdks prevents pRb from binding to E2F allowing E2F to activate the transcription of genes thought to be necessary for normal cell cycle progression.

Recently, two families of cyclin-dependent kinase inhibitors (CKIs) have been identified that inhibit cell cycle progression by binding to and inactivating cyclin/cdk complexes (Sherr and Roberts, 1995). The INK family, which includes p15\textsuperscript{INKdb}, p16\textsuperscript{INKda}, p18\textsuperscript{INKdc} and p19\textsuperscript{INKda} bring about G1 arrest by specific inhibition of D-cyclins complexed with cdk4 or cdk6. Mutations in p15 and p16 are prevalent in tumors, suggesting that they may normally act as tumor suppressor genes (Hunter and Pines, 1994). The p21 family of CKIs includes p21\textsuperscript{Waf1.Cip1.Sdi1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}. Although p21 and p27 can inhibit many cyclin/CDK complexes \textit{in vitro}, their \textit{in vivo} activity appears to be restricted to the G1 cyclin/CDK complexes (Sherr and Roberts, 1995). For example, transfection of these inhibitors arrests cells in the G1 phase of the cell cycle. As well p27 levels are typically highest in quiescent cells and decrease as cells enter the cell cycle. Although genetic alterations are common in INK family members, mutations in p21 and p27 appear to be rare in tumors (Ponce-Castaneda \textit{et al.}, 1995; Kawamata \textit{et al.}, 1995; Shiohara \textit{et al.}, 1994; Ferrando \textit{et al.}, 1996; Spirin \textit{et al.}, 1996) suggesting that tumor cells retaining such inhibitors may have a survival advantage.

\textit{1.3.3 Cyclin-Dependent Kinase Inhibitors and Drug Resistance}

Levels of the p27 cell cycle inhibitor have been shown to be elevated by cell-cell contact in both normal fibroblasts and epithelial cells in monolayer culture and has been proposed to regulate contact inhibition (Slingerland \textit{et al.}, 1994; Hengst \textit{et al.}, 1994; Polyak \textit{et al.}, 1994). In these studies
up to a 4-fold increase in p27 protein level was observed as cells reached confluence. Our studies described in chapter 3, demonstrate that p27 is also elevated by intercellular contact in tumor cells and is at least partly responsible for contact-dependent growth inhibition and resistance to anticancer agents.

Another CKI that shares structural and functional similarity to p27, and appears to regulate drug resistance is p21<sub>WAF1/CIP1</sub>. This CKI has been shown to prevent apoptosis (Wang and Walsh, 1996), and p21 homozygous negative (-/-) colon carcinoma cells are more sensitive to undergoing drug induced apoptosis than wildtype (+/+)) cells, while heterozygotes (+/-) display an intermediate phenotype (Waldman <i>et al.</i>, 1996). Importantly, the relative sensitivity of p21 -/- colon carcinoma cells has been confirmed in vivo using a tumor xenograft model (Waldman <i>et al.</i>, 1997). Furthermore, elevated p21 levels have been associated with chemo-resistance (Zhang <i>W. et al.</i>, 1995). Although p21 differs from p27 in that its levels are not regulated by cell-cell adhesion, both of these inhibitors share functional properties and both may prevent cells from undergoing drug-induced apoptosis.

1.3.4 Effects of Cell Adhesion on Signaling Pathways Regulating Growth

Cell adhesion molecules and extracellular matrix were once viewed simply as a "cellular glue" and/or mechanical scaffolding, primarily acting to hold cells together and providing structural support. However, it is now known that cell adhesion can have profound effects on cell behavior and can regulate processes such as cell growth, differentiation and survival (Juliano and Haskill, 1993). Exactly how adhesion initiates these changes in cell behavior at the molecular level has become a favorite topic of study for many cell biologists over the past several years. Although our
knowledge in this area remains sketchy, recent data promises to provide new insights into the signal transduction pathways regulated by cell adhesion (Hannigan and Dedhar, 1997).

Integrins represent the most well studied adhesion mechanism regulating signal transduction. Binding of integrins to the ECM has been shown to promote cell growth and survival, and antibodies which block integrin binding disrupt these processes (Meredith et al., 1993; Howlett et al., 1995; Strater et al., 1996; Rozzo et al., 1997). Integrins mediate the formation of focal adhesion plaques (FAP) which are sites of convergence between the ECM and actin stress fibers. Also found in FAPs are protein tyrosine kinases such as p125FAK, Src, and Csk. Integrin signaling may result from activation of p125FAK which becomes autophosphorylated upon integrin ligation. Recently, stimulation of cells with insulin has been shown to promote the association of αvβ3, integrin with insulin receptor substrate-1, an interaction that may potentiate insulin-mediated signaling (Vuori and Ruoslahti, 1994). As well, a novel integrin-linked serine/threonine protein kinase, p59ILK, has been shown to bind directly to the cytoplasmic domain of β1- and β3-integrins and may also be involved in integrin-dependent signal transduction (Hannigan et al. 1996).

Cell-cell contact mediated by CD44, N-cadherin and E-cadherin have been shown, like cell-ECM interactions mediated by integrins, to promote cell survival (Hermiston and Gordon, 1995a; Peluso et al., 1996; Henke et al., 1996; Ayroldi et al., 1995). However, cell-cell contact also leads to growth suppression or 'contact inhibition', whereas cell-ECM interactions generally promote cell cycle progression. Although suspected for decades, only recently has it been shown that cell adhesion molecules are in fact essential for contact inhibition. By adding to cell cultures neutralizing antibodies against NCAM or E-cadherin, for example, contact inhibition could be prevented in either fibroblasts or epithelial cells, respectively (Aoki et al., 1991; Kandikonda et al., 1996; Takahashi
and Suzuki, 1996). Likewise, adhesion mediated by vascular-endothelial (VE)-cadherin inhibits proliferation of endothelial cells (Caveda et al., 1996) and biliary glycoprotein 1, a member of the carcinoembryonic antigen family, inhibits the growth of both prostate and colon carcinoma cells (Hsieh et al., 1995; Kunath et al., 1995). Before our studies (see chapter 4) it was unclear exactly how adhesion molecules could inhibit growth although some interesting possibilities were suspected. Recent data have shown that β-catenin, in addition to linking cadherins to the actin cytoskeleton, functions in a signal transduction pathway initiated from the wnt proto-oncogene. Interest in this pathway increased substantially when β-catenin was shown to interact with transcription factors of the LEF/TCF family and affect gene transcription (Behrens et al., 1996; Huber et al., 1996; Molennar et al., 1996). It has been postulated that E-cadherin can sequester β-catenin at the cell surface and thereby prevent its signaling to the nucleus (Fagotto et al., 1996).

The second mechanism by which cell-cell contact has may inhibit growth involves regulation of the activity of growth factor receptors. It has been shown by several groups that ligand-induced phosphorylation of receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor or its related family member Erb-B2 is consistently lost in contact inhibited cells even in the presence of excess ligand (Takahashi and Suzuki, 1996; Mansbridge et al., 1992; Sorby and Ostman, 1996). In these studies phosphorylation of RTKs could only be rescued by the addition of phosphatase inhibitors, suggesting that cell-cell contact activates endogenous phosphatase activity of cells. In some cases cell-cell contact induced RTK downregulation (Suarez-quian and Byers, 1993), and reduced ligand binding affinity may also contribute to contact-dependent growth inhibition. However, these phenomena (unlike decreased RTK phosphorylation) have been inconsistent. Regardless of the exact mechanism(s), all such correlative studies suggest that cell-cell
contact results in inactivation of classical mitogenic pathways which in turn may be responsible for contact-dependent growth inhibition.

1.3.5 E-cadherin Mediates Contact-Dependent Growth Suppression

Although a number of studies have implicated cell-cell contact in the regulation of cell cycle kinetics, in most model systems the exact adhesion mechanism(s) responsible for contact-dependent growth inhibition remain elusive. Because adhesion mechanisms often differ between cell lines, numerous intercellular adhesion mechanisms may ultimately impinge on the same signal transduction pathways regulating cell cycle. E-cadherin, is ubiquitously found on normal epithelial cells. In a wide variety of human carcinomas including breast, gastric, head and neck, and prostate, E-cadherin expression is often lost or downregulated by mutation or by various epigenetic mechanisms (Siitonen et al., 1996; Berx et al., 1996; Becker et al., 1994). Gordon and co-workers have been able to abolish E-cadherin adhesion by transfecting cells with a dominant negative N-cadherin (DN-cadherin) which lacks a cytoplasmic tail. DN-cadherin presumably interacts with endogenous E-cadherin preventing it from binding to the underlying actin cytoskeleton. When targeted to the epithelial cells of the intestinal crypts, DN-cadherin leads to the formation of adenomas (Hermiston and Gordon, 1995b). Importantly, loss of E-cadherin function in crypt cells is associated with an increase in the rate of apoptosis (Hermiston and Gordon, 1995a; Hermiston and Gordon, 1995b). Inhibiting E-cadherin function in non-tumorigenic immortalized cell lines in culture with neutralizing E-cadherin antibodies prevents “contact inhibition” and results in extended growth and piling up of cells at high cell densities (Kandikonda et al., 1996; Takahashi and Suzuki, 1996). Despite these findings, not all carcinomas lose E-cadherin expression with progression, and
tumors can develop with an E-cadherin adhesion mechanism at least partially intact (Navarro et al., 1991; Ewing et al., 1995; Miyaki et al., 1995). Furthermore, when E-cadherin function is restored in tumor cells, tumor growth in vivo is slowed but not abolished (Bullions et al., 1997; Navarro et al., 1991; Ewing et al., 1995; Miyaki et al., 1995). These studies demonstrate that E-cadherin, traditionally described as an "invasion suppressor" (Vleminckx et al., 1991), is also a growth suppressor and its function is sometimes, but not always, lost with tumor progression.

1.4 Summary and Overview

The goal of this chapter was to introduce the clinical problem of drug resistance in the treatment of cancer and to summarize relevant research relating to possible mechanisms responsible for resistance at both the cellular and molecular level. Work in this area has led to the hypothesis that cell-cell adhesion may be a major negative regulator of growth, and this in turn may help to explain why tumors in a three-dimensional context generally display low growth fractions and are highly resistant to a wide variety of cell cycle dependent chemotherapeutic agents.

In chapter 2 experiments are described which assess the contribution of intercellular adhesion on multicellular drug resistance. The experiments used to address this question, arose from two fortuitous observations made concerning EMT-6 cells. First, by simply cloning the parent EMT-6 cell line by limiting dilution, it was possible to derive variants which spontaneously formed tightly-adherent or loosely adherent aggregates when plated in three-dimensional culture. Second, the nontoxic enzyme hyaluronidase was found to disperse EMT-6 tightly-adherent variants. These studies demonstrate that intercellular adhesion is associated with decreased growth and increased resistance to the anticancer agent cyclophosphamide. Hyaluronidase was used to demonstrate the
potential of using "anti-adhesives" as chemosensitizers in the treatment of tumor cells both in vitro and in vivo. Given that cyclophosphamide is known to preferentially target rapidly dividing tumor cells, we reasoned that intercellular adhesion may effect drug resistance indirectly through its effects on cell cycle.

The work described in chapter 3 is an investigation of the molecular mechanism regulating adhesion-dependent drug resistance. These studies implicate the cyclin-dependent kinase inhibitor p27 as one possible mediator of adhesion-dependent growth inhibition and of multicellular drug resistance. Adhesion-dependent upregulation of p27 may help to explain why tumor cells, despite harbouring many mutated oncogenes and tumor suppressor genes, still often contain low growth fractions in vivo. These studies also raise the possibility that p27 antagonists may be useful chemosensitizers in combination with current anticancer therapy.

In chapter 4 the question of whether contact-dependent growth inhibition can be mediated by more than one adhesion mechanism is addressed. These studies demonstrate that E-cadherin, like the previously uncovered but less defined hyaluronidase-sensitive adhesion mechanism of EMT-6 cells, can also inhibit proliferation. Furthermore, E-cadherin appears to signal growth inhibition through upregulation of p27. These studies may help to explain why carcinomas often lose E-cadherin function with tumor progression.

Finally, in chapter 5 work from the preceding chapters is discussed with a particular emphasis on the potential relevance of this work for understanding cancer biology in general, and why tumors are often resistant to conventional cytotoxic therapy. Highlighted are possible ways of circumventing drug resistance by combining conventional cytotoxic agents with novel chemosensitizers such as anti-adhesives and p27 antagonists.
Chapter 2

Reversal by hyaluronidase of adhesion-dependent multicellular drug resistance in mammary carcinoma cells

by: St. Croix B., Rak J.W., Kapitain S., Sheehan C., Graham C.H., and R.S. Kerbel

2.1 Abstract

**Background:** De novo or acquired resistance to chemotherapeutic drugs continues to be one of the most important obstacles hindering the successful treatment of cancer patients. Consequently, enhancing the efficacy of conventional chemotherapeutic drugs has become an important research goal. Our previous studies using the EMT-6 mouse mammary carcinoma selected for resistance to various alkylating agents *in vivo* demonstrated that such acquired drug resistance may be manifested *in vitro* only in cells growing in a three-dimensional configuration but not in conventional monolayer culture. We also found that this phenomenon, which we refer to as acquired "multicellular resistance," is associated with an increase in intercellular adhesion or compaction of the alkylating agent resistant cell lines grown as aggregates in three-dimensional culture.

**Purpose:** The present study further investigates the importance of three-dimensional architecture on multicellular drug resistance and its influence on cell cycle kinetics, cell cycle arrest and cell survival.

**Methods:** To test the hypothesis that an increase in three-dimensional compaction is related to the drug resistance of the cells, we did the following: 1) selected clones of the EMT-6 cell line that spontaneously formed tightly or loosely adherent aggregates and assessed their respective drug resistance *in vitro*; 2) assayed tumorigenic potential of the tight and loose clones after exposure to defined concentrations of the activated form of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC) *in vitro*; and 3) treated the tight clones with hyaluronidase, an agent capable of disrupting EMT-6 spheroids, and assayed the effect on chemosensitivity. We used flow cytometry to monitor any alterations in cell cycle kinetics.

**Results:** The increase in compaction in three-dimensional culture was sufficient to confer
resistance to 4-HC. This increase in intercellular adhesion was also associated with a reduced proliferating fraction of tumor cells and a lower proportion of cells arrested in G2/M following drug exposure. Furthermore, these changes were only detectable in three-dimensional culture, not in conventional monolayer culture. In conventional monolayer culture all cell types consistently showed a high level of proliferation and arrested in G2/M after exposure to 4-HC. Hyaluronidase was able to disrupt intercellular adhesion and chemosensitize tumor cells both in vitro and in vivo in an ascites model.

**Conclusion:** Earlier studies have demonstrated that hyaluronidase is able to sensitize tumor cells to various anticancer agents, and can do so in three-dimensional culture systems by increasing drug penetration into the spheroid core. Our studies now demonstrate that this sensitization can occur by a mechanism independent of increased drug penetration. This mechanism is likely to be related to the "anti-adhesive" effect of hyaluronidase, which overrides cell contact-dependent growth inhibition, recruits cells into the cycling pool, and renders tumor cells more sensitive to cytotoxic agents that preferentially kill rapidly dividing cells.

**Implications:** Other tumor-specific "anti-adhesives" should be explored. They may be effective chemosensitizers when used in combination with cell cycle specific drugs for the treatment of small, solid tumors.

### 2.2 Introduction

*De novo* or acquired resistance to chemotherapeutic drugs continues to be one of the most important obstacles hindering the successful treatment of cancer patients. Since current treatment modalities rely heavily on chemotherapeutic drugs and new cytotoxic agents discovered over the last
decade are seldom much better than those used historically, enhancing the efficacy of conventional drugs (chemosensitization) has become an important research goal. Hyaluronidase, although relatively non-toxic by itself, has been used as a chemosensitizer in combination with many chemotherapeutic drugs (Baumgartner et al., 1985; Liu et al., 1987; Baumgartner et al., 1988; Maier et al., 1989; Lehnert et al., 1989; Baumgartner et al., 1987; Beckenlehner et al., 1992; Kohno et al., 1994; Sprub et al., 1995). Its efficacy as a chemosensitizer in treating cancer patients was discovered serendipitously when a patient receiving chemotherapy for myeloma was unintentionally given a paravenous injection and then immediately given hyaluronidase at the same site (Baumgartner et al., 1985). (Hyaluronidase was also given, since it is known to speed up the resorption of cytostatic agents and to help prevent unwanted necrosis in normal tissues.) The unexpected improvement in this patient’s condition stimulated Baumgartner and co-workers to assess in more detail the potential of using hyaluronidase as a chemosensitizer (Baumgartner et al., 1985).

A limited number of experimental and clinical studies have now documented the benefits of combining hyaluronidase with chemotherapeutic drugs, and an encouraging phase I study (Liu et al., 1987) has shown that hyaluronidase is well tolerated and causes minimal increases in normal tissue toxicity. It is unclear, however, how hyaluronidase is able to enhance drug-induced cytotoxicity towards tumor cells, although increased drug diffusion in hyaluronic acid (HA)-rich regions of tumors has been proposed (Beckenlehner et al., 1992; Kohno et al., 1994).

In order to further rationalize the use of hyaluronidase and to optimize its efficacy or design alternative chemosensitizers, it would be of benefit to understand more fully how hyaluronidase is able to chemosensitize tumor cells to cytotoxic drugs. Many biochemical resistance mechanisms have been described, including, among others, reduced drug accumulation, increased drug efflux,
increased metabolic drug detoxification, and increased DNA repair. Almost all acquired drug resistance mechanisms described so far were uncovered by examining drug resistant variants derived by the continuous or stepwise drug-selection of cells growing in monolayer culture. However, it is still unclear what impact, if any, such mechanisms have on acquired clinical drug resistance which is often on the order of 3-4 fold. Furthermore, development of more effective treatment strategies based on knowledge of such drug resistance mechanisms has failed to live up to expectations, especially for solid tumors. (Sobrero and Bertino, 1986; Houghton and Kaye, 1994).

By use of drug-resistant cell lines selected for resistance in vivo, instead of in tissue culture, it may be possible to uncover mechanisms of resistance which have a greater probability of explaining the basis of clinical drug resistance in solid tumors (Teicher et al., 1990; Starling et al., 1990; Schecter et al., 1991). In this regard, Teicher et al. generated a series of alkylating agent-resistant variants of the EMT-6 mouse mammary carcinoma by repeatedly exposing syngeneic, tumor-bearing BALB/c mice to various cytotoxic agents (Teicher et al., 1990). Although highly resistant in vivo, the drug selected variants unexpectedly lost their resistant phenotype when cells were grown in conventional monolayer culture (Teicher et al., 1990). Subsequently, our laboratory (Kobayashi et al., 1993) found that the dmg resistant properties of these cells could be "rescued" in vitro if the cells were grown under three-dimensional culture conditions (i.e., as multicellular tumor spheroids). This resistance, which we refer to as "acquired multicellular resistance," was also associated with an increase in intercellular adhesion (or compaction) of the drug resistant variants compared with the loosely adherent EMT-6 parental cell line (EMT-6/P). In another study (Graham et al., 1994), using both the mouse EMT-6/P and human MDA-MB-231 breast cancer cell lines, we have shown that a transient increase in both aggregate compaction and drug resistance can occur
rapidly following exposure to 4-hydroperoxycyclophosphamide (4-HC), an activated form of cyclophosphamide.

The purpose of the present study was to determine whether or not this increase in intercellular adhesion observed in three-dimensional culture was sufficient to confer resistance to 4-HC. Because hyaluronidase was able to disrupt this type of compaction in EMT-6 mouse mammary tumor cells, it was used as a tool to address this question. In addition, in a series of experiments, we exposed randomly selected cellular variants displaying different degrees of adhesion in three-dimensional culture to 4-HC and then examined the cells for changes in cell cycle kinetics, cell cycle arrest, and survival. We conclude that intercellular adhesion plays a causal role in resistance of solid tumor cells to 4-HC, possibly through alterations in cell cycle kinetics.

2.3 Materials and Methods

2.3.1 Tumor cell lines and cell culture

The EMT-6 mouse mammary tumor cell line (EMT-6/P) and its in vivo derived alkylating-agent-resistant variants (previously referred to as EMT-6/CTX and EMT-6/CDDP but hereafter referred to as E/CTX and E/CDDP) (Teicher et al., 1990) were a gift from Dr. Beverly Teicher of the Dana-Farber Cancer Institute, Boston, MA. EMT-6 multicellular aggregates were prepared by the liquid overlay technique, as previously described (Kobayashi et al., 1993). All EMT-6 cells were cultured in Waymouth’s MB 752/1 medium (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂ and 95% air. Our panel of clones that form "tightly" or "loosely" adherent cellular aggregates were obtained by cloning the EMT-6/P cell line by limiting dilution, expanding and then plating the clones into three-dimensional culture,
and selecting the most extreme clones which displayed either a tight (compact) or loose morphology. The letter "T" or "L" following the designated name of each clone was used to indicate the "tightly" or "loosely" morphology of the clone, respectively; the lower-case letter "c" with a number indicates the clone number. Clones retained their respective adhesive phenotypes for at least 2-3 months in culture. For all experiments reported here, frozen stocks less than one month old were used.

2.3.2 *In vitro colony formation assay*

In order to allow maximal and uniform drug exposure, cells growing in monolayer culture were treated with various concentrations (6-100 \( \mu \text{M} \)) of 4-HC for 1 hour, after which cells were rinsed, trypsinized, and replated in three-dimensional culture. In the case of the hyaluronidase experiments, drug-treated cells were simultaneously replated back into monolayers or into spheroid culture and grown in the presence or absence of hyaluronidase. After three days of culture under these conditions, cells were rinsed again, trypsinized and plated at various dilutions in a colony formation assay (Kobayashi et al., 1993). In the time course experiment, cells were exposed to 50\( \mu \text{M} \) 4-HC in monolayer culture and left for 24, 48 or 72 hour in three-dimensional culture before plating in the colony formation assay. Surviving fraction (S.F.) was calculated using the formula: 

\[
\text{SF} = \text{Plating Efficiency (P.E.) of the drug treated group/P.E. of the non-treated control.}
\]

Average values ± standard deviations from triplicate well were used to calculate P.E. These experiments were repeated at least three times with similar results.

2.3.3 *In vivo tumorigenicity assay*

An *in vivo* tumorigenicity assay was designed to test resistance to 4-HC. Cells from tight
or loose clones growing in three-dimensional culture for two days were exposed to 15 μM 4-HC; 24 hours later, they were rinsed with phosphate-buffered saline (PBS), trypsinized, and 5 x 10⁶ cells were injected subcutaneously into syngeneic BALB/c mice. The same experimental procedure was used in the hyaluronidase experiments, except that cells of one of the tight clones, E/Pc5-T, were maintained in three-dimensional culture in complete medium containing 2 mg/ml of hyaluronidase. Tumor volume (V) was calculated using the formula \( V = \frac{d^2D}{2} \) where \( d \)= the smallest tumor diameter (mm) and \( D \) = the largest tumor diameter (mm).

2.3.4 Bromodeoxyuridine (BrdU) incorporation assay

Cells growing in either three-dimensional culture for two days or in monolayer culture were pulsed with 10 μM BrdU for three hours, after which cells were immediately rinsed with PBS, trypsinized, rinsed again, and fixed with 70% ethanol (4°C) for at least 1 hour. Cells were rinsed of ethanol with PBS, pelleted, and resuspended in 1 ml of cold 0.1 N HCl-0.7% Triton X-100, and left for 10 minutes on ice. After adding 2 ml of PBS, centrifuging the mixture (5 minutes, 1000 rpm, 4°C) and decanting the supernatant, cells were resuspended in 0.5 ml of distilled H₂O and transferred into a 0.5-ml Eppendorf tube already containing 16 μl of 0.1 N HCl. Next, the Eppendorf tubes were heated in a polymerase chain reaction machine for 8 minutes at 95°C, and then placed immediately on ice for 10 minutes. The cells in suspension were transferred to a 5-ml tube, rinsed two times with HNFN buffer (i.e. 10 mM HEPES [pH7.4], 150 mM NaCl, 4% FCS, 0.1% NaN₃) and once with HNFN-0.5% tween 20, and the cell pellets were resuspended in 200 μl of a 1/40 dilution of fluorescein isothiocyanate-anti-BrdU antibody (Cedarlane Laboratories Ltd., Hornby, ON) and left in the dark on ice for 45 minutes. Stained cells were rinsed twice with HNFN and once with HNFN-
tween 20, pelleted, resuspended in 1 ml of PI solution (i.e. 50 μg/ml of propidium iodide and 10 μg/ml of ribonuclease A in PBS) and left for 1 hour (4°C) in the dark before they were analyzed on an Epics Elite V flow cytometer (Coulter Electronics, Burlington, ON).

2.3.5 Disruption of intact spheroids using hyaluronidase

Addition of hyaluronidase to the medium could prevent cell aggregation or disrupt pre-formed compact spheroids even after 2 or more days of growth in three-dimensional culture. In all experiments reported here, 2 mg/ml of bovine testicular hyaluronidase (Sigma Chemical Co., Mississauga, ON) was used because it was nontoxic and highly effective at disrupting spheroids.

2.3.6 Monitoring cell cycle perturbations following 4-HC exposure

Exponentially growing monolayer cultures or two day old three-dimensional cultures were exposed to 15 μM 4-HC and left in the presence of drug for 48 hours. In other experiments, cells maintained in the presence or absence of hyaluronidase were exposed to various concentrations of drug for 24 hours or followed over time and then collected. To block the cells at the G2/M phase of the cell cycle, 1 μg/ml of Nocodazole (Sigma Chemical Co.) in dimethyl sulfoxide (DMSO) or (as a control) DMSO alone (0.08% vol/vol) was added to the growth medium at the time cells were plated in three-dimensional culture. For DNA analysis, cells were rinsed with PBS, trypsinized, fixed in 70% ethanol for at least 1 hour, rinsed again with PBS, filtered through 30-μm mesh, and then stained with PI solution.
2.3.7 In vivo survival assay

E/Pc5-T cells (5 x 10^6) were injected intraperitoneally into BALB/c mice. On days 1, 3, and 5 after injection, control mice received an intraperitoneal injection of either 20,000 U of bovine testicular hyaluronidase in 0.5 mL of PBS or PBS alone. Drug-treated mice were given the same regimen (ie. hyaluronidase or PBS) combined with 150 mg/kg of cyclophosphamide (given intraperitoneally in PBS); they received three additional treatments on days 11, 13, and 15 (i.e., drug-treated mice received combined treatments on 6 different days). Differences in survival between drug-treated groups were analyzed statistically by use of the Mann-Whitney test.

2.4 Results

2.4.1 Morphology of Cellular Aggregates of Spontaneously Selected Clones of the EMT-6 Cell line

In this study, we were interested in determining if the increase in three-dimensional compaction that we have observed in mouse EMT-6 mammary carcinoma cells following drug selection in vivo (Kobayashi et al., 1993) was in any way related to the drug resistance properties of these cells. We hypothesized that the EMT-6 parental population may be heterogeneous with respect to adhesion and that drug exposure could result in the selection of the more resistant "tight" variants. To test this hypothesis, we generated a series of clones of the EMT-6/P cell line. These clones displayed either a tight (compact) or loose morphology when grown in three-dimensional culture but had never been exposed to drug (Fig. 2.1). To derive such variants, approximately 200 EMT-6/P random clones obtained by limiting dilution were expanded and plated in three-dimensional culture by use of the liquid overlay technique to assess their spheroid forming ability. We observed that approximately 70% of the clones formed loose, irregular aggregates (e.g., E/Pc10-
Figure 2.1 Selection of clones of the EMT-6 parental cell line (EMT-6/P) displaying tight or loose morphology in three-dimensional culture. Clones of the EMT-6 cell line, obtained by limiting dilution, were expanded and plated into three-dimensional culture to assess their spheroid-forming ability. The tightest and loosest clones were retained, and 10,000 cells of each were plated onto 96-well plates pre-coated with agarose. After 2 days of growth under these conditions, aggregates were photographed under 40x magnification (Bars = 0.5 mm).
L, E/Pc11-L, and E/Pc14-L) indistinguishable in morphology from the EMT-6/P cell line (Fig. 2.1, upper panel), while approximately 25% were more adherent with some variability. Notably, about 5% of the clones looked identical to the alkylating agent-resistant variants described previously, i.e., formed very compact spheroids (e.g. E/Pc5-T, E/Pc7-T, and E/Pc11-T; Fig. 2.1, lower panel) (Kobayashi et al., 1993) and were therefore maintained along with the loosest clones for further analysis.

2.4.2 Increases in Colony-Forming Ability of EMT-6 Tight Clones After Exposure to 4-HC

We designed an in vitro colony formation assay to measure the survival capacity (clonogenic ability) of our tight and loose clones. Although previous studies (Durand, 1986; Durand, 1989) indicated that various alkylating agents have little difficulty in penetrating spheroids, limited drug penetration in compact spheroids has been reported to be a mechanism of resistance for certain other high-molecular-weight chemotherapeutic agents (Durand, 1986; Durand, 1989; Durand, 1990; Kerr and Kaye, 1987; Erlanson et al., 1992). Therefore, we analyzed whether or not resistance could be detected in our system in the absence of any potential barrier to drug penetration. The assay was designed such that it allowed us to compare the drug resistance properties of tight and loose clones exposed to the same concentration of drug. First, tight and loose clones were exposed to 4-HC for 1 hour in monolayer culture (where all cells would be exposed to drug), after which drug was rinsed away; cells were then harvested and plated onto agarose-coated, 24-well dishes, upon which cells spontaneously formed tight or loose aggregates. After two days of growth in three-dimensional culture, tight and loose clones were harvested again and plated in a standard colony formation assay. The results obtained suggest that the tight clones were much more resistant to 4-HC than the loose
clones (approximately 10-fold at 50μM 4-HC), even when exposed to drug under identical conditions (Fig. 2.2A).

2.4.3 Protection of EMT-6 Tight Clones From 4-HC-Induced Toxicity in a Tumorigenicity Assay

To verify our in vitro colony formation data, we designed an in vivo tumorigenicity assay that involved exposing the cells growing in three-dimensional culture to 15 μM 4-HC and injecting 5 x 10^5 cells subcutaneously 24 hours later into syngeneic BALB/c mice. This assay allowed us to measure the tumorigenic capacity of tumor cell populations following exposure to defined drug concentrations in vitro. As shown in Fig. 2.2B, no obvious differences in the in vivo growth properties of tight or loose clones was observed in the non-treated controls. In contrast, in the group treated with 15 μM 4-HC, all of the tight clones gave rise to tumors much more rapidly than the loose clones, with very little growth delay over non-treated controls. Furthermore, tumors formed in all mice given an injection of tight clones, whereas only a partial tumor take was observed in those mice receiving loose clones. The E/CTX cyclophosphamide-resistant cell line which was also included as a positive control displayed an even higher tumorigenic capacity than the tight clones.

2.4.4 Cell Cycle Changes of EMT-6 Clones After Exposure to 4-HC

We hypothesized that an increase in adhesion acquired either spontaneously or after drug exposure, by inhibiting cell proliferation, may render cells more resistant to cytotoxic agents known to be most effective against rapidly dividing cells. To assess this possibility, we analyzed the cell cycle kinetics of our panel of tight and loose forming clones. As shown in Table 2.1, when pulsed with BrdU after 2 days of growth in three-dimensional culture, cells of the loose clones incorporated
Figure 2.2 A) *In vitro* survival of EMT-6 tight and loose clones after exposure to 4-hydroperoxycyclophosphamide (4-HC). Clones exposed to 4-HC in monolayer for 1 hour were dispersed, replated into three-dimensional culture where they were left for two days, and then plated into a colony formation assay.
Figure 2.2 B) *In vivo* tumorigenicity of EMT-6 tight and loose clones after exposure to 4-hydroperoxycyclophosphamide (4-HC) *ex vivo*. Cells (5 x 10^5) growing in three-dimensional culture either untreated or exposed to 15μM 4-HC for 24 hours were dispersed and then injected (5 x 10^5) subcutaneously into BALB/c mice. All mice receiving an injection of non-drug-treated control cells developed tumors (n=7). The ending "T" or "L" indicates the clones’ tight or loose morphology, respectively. Values shown are the average ± standard error.
Table 2.1  Proliferation of EMT-6 cell lines (% BrdU labelled cells following 3 hour pulse)‡

<table>
<thead>
<tr>
<th></th>
<th>EMT-6/P*</th>
<th>E/Pc10-L*</th>
<th>E/Pc11-L*</th>
<th>E/CTX†</th>
<th>E/Pc5-T†</th>
<th>E/Pc7-T†</th>
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<tbody>
<tr>
<td>2D</td>
<td>60.4</td>
<td>71.7</td>
<td>68.6</td>
<td>56.0</td>
<td>59.2</td>
<td>57.8</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>63.9</td>
<td>71.0</td>
<td>69.7</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>3D</td>
<td>27.8</td>
<td>41.4</td>
<td>51.5</td>
<td>13.2</td>
<td>12.8</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>32.0</td>
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<td>36.8</td>
</tr>
<tr>
<td>HYase</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

2D = Two-dimensional monolayer culture

3D = Three-dimensional spheroid culture

ND = Not determined

HYase = Hyaluronidase treated

* loosely adherent cell lines

† tightly adherent cell lines

‡ this experiment was repeated at least 3 times with similar results
twofold to fourfold more label than cells of the tight clones.

Exposure of tumor cells, growing either in monolayer culture or as single-cell suspensions, to a number of chemotherapeutic agents (including 4-HC) is known to induce a G2/M cell cycle arrest (Bullock et al., 1993; Davidoff and Mendelow, 1993). We were interested in analysing cell cycle changes following exposure of cells to 4-HC and comparing results obtained from cells grown as monolayers or as three-dimensional tight or loose aggregates. As expected, when exposed to 15 μM 4-HC in monolayer culture, all of our EMT-6 variants were arrested at the G2/M phase of the cell cycle by 48 hours (data not shown; but see Fig. 2.4A). Likewise, a significant G2/M arrest was observed in all of the loose clones exposed to 15 μM 4-HC and left in the presence of drug for 48 hours in three-dimensional culture (Table 2.2). In contrast, the clones displaying tight morphology appeared to be relatively resistant to 4-HC-induced cell cycle perturbations (i.e., only a small fraction of cells were arrested in G2/M phase following drug exposure compared to loosely adherent controls). It was notable that the resistant sublines E/CTX and E/CDDP were also highly resistant to cell cycle changes in three-dimensional culture (data not shown). Thus, large differences in cell cycle arrest between tightly and loosely adherent cells after exposure to 4-HC were detectable only when the various tumor cell lines were grown in three-dimensional culture, not in conventional monolayer culture.

2.4.5 Role of Hyaluronidase in Prevention of Compaction, Alteration of Cell Cycle Kinetics, and Sensitization of Tumor Cells to 4-HC

Since an increase in adhesion in three-dimensional culture appears to be sufficient to confer resistance to 4-HC, we were interested in determining if this type of resistance could be reversed by
Table 2.2  Cell cycle perturbations 48 hours following exposure to 15 μM 4-Hydroperoxycyclophosphamide in three-dimensional culture ‡

<table>
<thead>
<tr>
<th></th>
<th>%G1</th>
<th></th>
<th>%S</th>
<th></th>
<th>% G2/M</th>
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<td></td>
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<td>47.7</td>
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<td>4.7</td>
<td>58.6</td>
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<td>64.4</td>
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<td>19.0</td>
<td>4.8</td>
<td>16.6</td>
</tr>
<tr>
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<td>3.5</td>
<td>12.3</td>
</tr>
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<td>81.6</td>
<td>15.6</td>
<td>8.9</td>
<td>3.6</td>
<td>9.5</td>
</tr>
<tr>
<td>E/CTX + HYase*</td>
<td>64.9</td>
<td>15.6</td>
<td>21.5</td>
<td>16.6</td>
<td>13.6</td>
<td>67.8</td>
</tr>
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<td>42.1</td>
<td>3.8</td>
<td>35.8</td>
<td>27.4</td>
<td>22.1</td>
<td>68.8</td>
</tr>
<tr>
<td>E/Pc7-T + HYase</td>
<td>51.2</td>
<td>0.8</td>
<td>31.0</td>
<td>10.8</td>
<td>17.8</td>
<td>88.4</td>
</tr>
</tbody>
</table>

HYase = Hyaluronidase treated

* loosely adherent cell lines

† tightly adherent cell lines

‡ this experiment was repeated at least 3 times with similar results
inhibiting intercellular adhesion. We began by investigating the mechanism of adhesion responsible for compaction, but were unable to identify any obvious differences in the expression of a number of known adhesion molecules between our tight and loose cell lines. E-cadherin, for example, was not detectable by Western blot or FACS analysis. Other adhesion molecules, such as the "promiscuous" \( \beta_1 \) integrin or the epithelial integrin \( \alpha_6\beta_4 \) were detected (by Northern blot and FACS analysis, respectively) but were expressed at similar levels between the various cell lines. CD44, a major cell adhesion molecule known to bind hyaluronan, was also detected; however, if anything, EMT-6 variants of loose morphology expressed somewhat higher levels of mRNA and protein than those of compact morphology (data not shown). Next, we tried enzymatic means to disrupt adhesion. Although pure preparations of specific enzymes such as collagenase had no effect, bovine testicular hyaluronidase was able to disrupt completely adhesion of compact, preformed spheroids (Fig. 2.3). This activity appeared to be specific as pure \textit{Streptomyces hyalurolyticus} hyaluronidase (ICN Biomedicals Inc., Costa Mesa, CA) was also able to disrupt compact preformed spheroids when used in serum-free medium (data not shown). Concomitant with its effects on adhesion, hyaluronidase also increased the proliferation of tight clones in three-dimensional culture to a level similar to that of the loose clones (see Table 2.1).

We subsequently evaluated the effect of spheroid dissociation by hyaluronidase on cell cycle kinetics after exposure to 4-HC. As shown in Fig. 2.4 and Table 2.2, spheroids of tight clones dissociated in this manner following exposure to 4-HC showed a large G2/M arrest. The kinetics with which cells accumulated in G2/M appeared to be dose dependent, and an S-phase delay was observed at the higher drug concentrations (Fig. 2.4A). In contrast to the hyaluronidase-disrupted cells, we only observed a small accumulation of G2/M cells in either spontaneous or drug-resistant,
Figure 2.3 Disaggregation of compact E/CTX and E/Pc7-T spheroids using hyaluronidase. E/CTX cells (A and B) or E/Pc7-T cells (C and D) were plated in 100µL of complete growth medium in the presence (B and D) or absence (A and C) of 2 mg/ml hyaluronidase. All photographs were taken under 40x magnification after 2 days of growth in three-dimensional culture. (Bars = 0.5 mm)
Figure 2.4 A) Cell cycle perturbations of E/Pc5-T cells 24 hours after exposure to 4-hydroperoxycyclophosphamide (4-HC) in the presence or absence of hyaluronidase. E/Pc5-T cells growing in three-dimensional culture for 2 days or in monolayer culture in the presence or absence of hyaluronidase (HYase) were exposed to various concentrations of 4-HC for 24 hours and then collected for cell cycle analysis. The abscissa represents relative DNA content, and the ordinate represents relative cell number.
compact spheroids, even when cells were exposed to up to 50 μM (Fig. 2.4A) or even 100 μM 4-HC (data not shown). We reasoned that cells in tight spheroids may be cycling more slowly than those in loose, hyaluronidase-treated aggregates (see Table 2.1), and consequently it may take longer for the adherent cells in spheroids to arrest in G2/M following drug exposure. However, a time course revealed very little change in cell cycle parameters in tightly adherent variants beyond 48 hours in three-dimensional culture (Fig. 2.4B). It is noteworthy that a proportion of cells from compact spheroids continue to cycle through G2/M following drug exposure since addition of the M-phase blocker Nocodazole to the medium consistently resulted in an ~15% increase in the fraction of cells arresting in G2/M following drug exposure (Fig. 2.4C). In sharp contrast to the results obtained with compact spheroids, cells from loose aggregates treated with 4-HC and hyaluronidase continued to arrest in G2/M, such that by 96 hours virtually no cells were detectable in any other phase of the cell cycle. By continuously changing the medium, we have followed these cells for more than 2 weeks and have never observed the reappearance of a normal cell cycle profile. We did note, however, that a large proportion of this hyaluronidase-treated population eventually becomes tetraploid (data not shown).

To evaluate the effect of hyaluronidase on adhesion dependent 4-HC resistance, once again, we used our in vitro colony formation assay, which allowed us to measure cell survival in the absence of any potential barrier to drug penetration (Fig. 2.5A). The assay involved exposure of cells growing in monolayer culture to 4-HC for one hour and then harvesting and replating them in either monolayer culture or three-dimensional culture in the presence or absence of hyaluronidase. After 3 days of further growth under these conditions, cells were again harvested and plated in a colony formation assay. The results obtained show that the most resistant cells were those
Figure 2.4 B) Time course demonstrating kinetics of cell cycle perturbations of E/Pc5-T cells after exposure to 4-hydroperoxycyclophosphamide (4-HC) in the presence or absence of hyaluronidase. E/Pc5-T cells, growing in three-dimensional culture in the presence or absence of HYase for 2 days, were exposed to 15 μM 4-HC and then collected at various times thereafter for cell cycle analysis. Percentages of cells in each phase of cell cycle were calculated by use of the multicycle software program. The data shown are representative of three independent experiments.
Figure 2.4 C) Effect of Nocodazole treatment on G2/M arrest of E/Pc5-T cells following exposure to 4-hydroperoxycyclophosphamide (4-HC). E/Pc5-T cells growing in three-dimensional culture in the presence or absence of HYase for 2 days, were exposed to 15mM 4-HC and then collected at various times thereafter for cell cycle analysis. At the time of drug exposure, Nocodazole in dimethyl sulfoxide (DMSO) or DMSO alone (control) was also added to the growth medium. Percentages of cells in each phase of cell cycle were calculated by use of the multicycle software program. The data shown are representative of two independent experiments.
Figure 2.5 A) Hyaluronidase (HYase) decreases cell survival after exposure of E/Pc5-T cells to 4-hydroperoxycyclophosphamide (4-HC) in vitro. E/Pc5-T cells exposed to 4-HC in monolayer for 1 hour were dispersed and replated into either monolayer (2-D) or three-dimensional (3-D) culture in the presence or absence of HYase. After 3 days, cells were dispersed and plated into a colony formation assay (CFA). For the time course (inset), cells exposed to 50μM 4-HC in monolayer culture were replated and left in three dimensional culture for 24, 48 or 72 hours before plating in a CFA.
maintained in three-dimensional culture in the absence of hyaluronidase (tight aggregates), followed by those grown in the presence of hyaluronidase (loose aggregates). As reported for many other DNA damaging agents (Hoffman RM, 1993; Miller et al., 1985; Kwok and Twentyman, 1985; Dertinger and Hulser, 1981; Sutherland, 1988; Olive et al., 1993), cells growing in monolayer culture were much more sensitive to 4-HC than cells growing in three-dimensional culture. The slight increase in drug sensitivity seen in monolayer cultures in the presence of hyaluronidase may be due to the slight increase in cell proliferation observed following exposure to hyaluronidase (see Table 2.1). Because some cell-cell contact is maintained in monolayer culture, it is possible that, even under these conditions, hyaluronidase may increase proliferation by decreasing adhesion.

The results from our colony formation assay revealed that hyaluronidase sensitized cells to 4-HC, especially in three-dimensional culture. We were interested in determining how much time was required for this sensitivity to develop. We, therefore, analyzed the colony forming ability of cells 24, 48 and 72 hours after exposure to 4-HC in three-dimensional culture (see inset Fig. 2.5A). As expected, cells growing in hyaluronidase-containing medium were always more sensitive than cells growing in its absence. To our surprise, however, differences in the level of resistance remained relatively constant over the time course, although the number of clonogenic cells increased at a relatively steady rate. This result suggests that the presence of hyaluronidase in the medium during the initial 24 hours after drug exposure is critical for its chemosensitizing activity (compare with results shown in Fig. 6). In the presence or absence of hyaluronidase, growth in three-dimensional culture after exposure to 4-HC by itself appears to “rescue” cells from otherwise imminent drug-induced loss of clonogenic ability.

As an independent method of measuring the effect of hyaluronidase on 4-HC resistance, we
used our assay to measure changes in overall tumorigenicity *in vivo* after exposure of tumor cells to cytotoxic agents *in vitro*. In this case, the tight clone E/Pc5-T was grown in three-dimensional culture for 2 days in the presence or absence of hyaluronidase and exposed to various concentrations of 4-HC; 24 hours later, $5 \times 10^5$ cells were injected subcutaneously into BALB/c mice. As shown in figure 2.5B, pre-exposure to hyaluronidase *in vitro* did not affect the subsequent growth of non-treated controls *in vivo*. When E/Pc5-T cells were treated with 4-HC, however, notable differences in tumor take and latency period were observed, depending on whether or not hyaluronidase was included in the medium. If hyaluronidase was present, for any given drug concentration, tumor latency was longer and tumor take was less than that observed for cells grown identically except in the absence of hyaluronidase. These changes appeared to be dose dependent such that, at a concentration of 12 $\mu$M 4-HC, E/Pc5-T cells gave rise to tumors only if these cells were grown in the absence of hyaluronidase before injection.

These results stimulated us to determine whether hyaluronidase could also act as a chemosensitizer for cyclophosphamide when both agents are administered simultaneously to mice bearing EMT-6 tumors. For this purpose, we decided to grow EMT-6 tumor cells as ascites, reasoning that such free-floating cellular aggregates may retain properties reminiscent of those observed in three-dimensional suspension culture. To visualize if this hypothesis was true, the tight clone E/Pc5-T growing as an ascites tumor with or without pre-treatment with hyaluronidase was removed and analyzed. While many large "spheroid-like" clusters were observed in the untreated group, similar clumps were not found in the hyaluronidase-treated mice (Fig. 2.6A). Next we analyzed the effect of hyaluronidase on survival of drug-treated, tumor-bearing mice. E/Pc5-T ascites tumors were treated repeatedly with hyaluronidase or PBS either alone (controls) or in
Figure 2.5 B) Hyaluronidase (HYase) decreases tumorigenicity after exposure of E/Pc5-T cells to 4-hydroperoxycyclophosphamide (4-HC) in vitro. In vivo tumorigenicity was measured by exposure of the tightly adherent clone E/Pc5-T growing in three-dimensional culture to 0, 6, or 12 µM 4-HC in either the presence or absence of HYase. Twenty-four hours later, 5 x 10^5 cells were injected subcutaneously into BALB/c mice. Values shown are the averages ± standard error. Arrowhead pointing down indicates "out of scale" data point for the open triangle (Δ), since no tumors arose in this group.
Figure 2.6 A) Hyaluronidase (HYase) disperses E/Pc5-T tumor cells *in vivo*. E/Pc5-T ascites tumors were removed and visualized 24 hours after HYase treatment and 5 days after injection. Note the absence of clumps in the HYase-treated (+HYase) mice. (Bar = 0.5 mm)
combination with 150 mg/kg cyclophosphamide, the maximum tolerated dose (drug-treated). Although hyaluronidase by itself did not appear to affect survival time significantly, when it was administered in combination with cyclophosphamide, a significant increase in survival was noted ($P<.005$) (Fig. 2.6B). The median survival time was only 28 days in the group treated with cyclophosphamide alone, but when cyclophosphamide was administered together with hyaluronidase, half of the mice were still alive at day 47. Thus, hyaluronidase also inhibits adhesion and acts as a chemosensitizer for EMT-6 cells in vivo.

2.5 Discussion

Our study shows that an increase in three-dimensional compaction of multicellular tumor spheroids is indeed sufficient to confer resistance to the alkylating agent 4-HC and that hyaluronidase can reverse compaction and sensitize tumor cells to both 4-HC in vitro and to cyclophosphamide in vivo. Thus, adhesive cell interactions may play an important role in both intrinsic and acquired multicellular resistance to alkylating agents. If so, understanding the molecular mechanisms regulating cellular aggregation is of critical importance. The disaggregating effect of hyaluronidase implies the involvement of HA and HA receptors. On the basis of recent experiments, we now believe that there are at least two independent adhesion processes that take place in three-dimensional culture. The initial adhesion event occurs rapidly (within 30 minutes) when cells are placed in three-dimensional culture and is probably shared between the various EMT-6 cell lines. This adhesion appeared weak, inasmuch as cells were easily dispersed from such aggregates by mechanical pipetting (unpublished observation). By staining loosely or tightly adherent variants with nontoxic fluorescent dyes and then mixing the two together and plating them
Figure 2.6 B) Hyaluronidase (HYase) increases survival time of tumor bearing mice following cyclophosphamide treatment in vivo. E/Pc5-T cells were injected intraperitoneally on day 0 and at various time points thereafter (arrows) were treated with the regimens indicated. Drug-treated mice received 6 treatments in total, whereas non-drug-treated controls received only the first 3. The single surviving mouse of the group treated with both HYase and cyclophosphamide (CTX) eventually died on day 72 (n=10 for drug-treated and n=5 for non-drug-treated groups).
in three-dimensional culture, we have observed that, by six hours, the tightly adherent cells are able to recognize and homotypically bind to one another. This secondary "homotypic" adhesion mechanism appears to be much stronger than the first, since by 48 hours it is not possible to disperse such clumps mechanically under the same conditions or in the presence of EDTA (unpublished observations). We believe that this latter adhesion mechanism, which is hyaluronidase sensitive and divalent cation independent, is responsible for the compaction we have observed in the drug-resistant variants and tight clones.

The exact molecular mechanism(s) responsible for compaction of our EMT-6 variants in three-dimensional culture is unknown. The simplest model one can propose is that HA binds to receptors on adjacent cells, thereby forming a molecular bridge which holds compact variants together, and that hyaluronidase, by degrading HA, abolishes this intercellular adhesion. In this regard, Underhill and Toole showed that various fibroblast lines transformed with simian virus 40 acquire the ability to bind endogenous HA on adjacent cells using cell surface HA receptors (Underhill and Toole, 1981; Underhill, 1982). CD44 has been shown to be the major HA receptor (Aruffo et al., 1990), and it is found on a surprisingly large variety of cell types. The fact that CD44H, the standard form of CD44, appears to be expressed at a slightly higher level in our loosely versus tightly adherent clones may still be consistent with the cross-bridging model if one considers that, in the loose clones, all of the binding sites for HA may be saturated by surface bound HA thus preventing cross-bridging from occurring. This hypothesis remains to be tested (for more information on the HA-dependent adhesion mechanism of EMT-6 cells see section 5.1, page 111).

It is interesting that hyaluronidase can increase the growth of our tightly adherent EMT-6 variants, particularly in three-dimensional culture. It is possible that hyaluronidase releases growth
factors bound to HA (Ruoslahti and Yamaguchi, 1991) or releases small hyaluronic acid fragments that have been shown to stimulate the growth of certain cell types (Goldberg and Toole, 1987). However, the fact that a similar increase in growth rate can be achieved by selecting for EMT-6 clones that spontaneously form loose aggregates suggests that loss of cellular adhesion per se may constitute a signal regulating cell division. Moreover, conditioned medium from the loose EMT-6 variants did not increase the growth or cause disaggregation of our tight variants (unpublished observations). It is notable that preferential expression of CD44 and increased HA synthesis have been observed on rapidly proliferating cells (Alho and Underhill, 1989; Brecht et al., 1986). The HA-binding region of CD44 as well as RHAMM (receptor for HA-mediated motility), another cell surface HA receptor, seems to be essential for the tumorigenic or metastatic properties of various tumor cell lines (Bartolazzi et al., 1994; Sy et al., 1991; Sy et al., 1992; Birch et al., 1991; Zhang et al., 1995; Hall et al., 1995). Collectively, these studies demonstrate the importance of HA for tumor growth and suggest its possible contribution to adhesion-dependent drug resistance in certain tumors or cell lines.

One of the objectives of the present study was to investigate possible mechanisms by which compaction may contribute to the adhesion-dependent, multicellular drug resistance phenotype. We began by analysing cell cycle perturbations following drug exposure, since such changes have been assessed only rarely in a solid tumor-like, three-dimensional context (Sano et al., 1983). It is surprising that compact spheroids were extremely resistant to 4-HC-induced G2/M arrest, even when they were given supraphysiological concentrations of drug and left for extended periods of time in three-dimensional culture. It is possible that a proportion of these cells, particularly those near the necrotic core of the spheroid, have become quiescent, thus making them relatively unresponsive to
4-HC. However, notable S and G2/M fractions in untreated spheroids suggest that a proportion of spheroid cells, most likely those cells nearest the outer periphery, are cycling. Treatment with the M-phase blocker Nocodazole resulted in an enhanced G2/M arrest in tightly adherent spheroids following 4-HC exposure. Thus, some cells of compact spheroids continue to cycle following drug treatment but are resistant to cell cycle perturbations. Our finding is consistent with the observations of Sano and coworkers (Sano et al., 1983; Deen et al., 1979), who have shown a similar correlation in spheroids versus monolayers of 9L cells after exposure to the alkylating agent Spirohydantoin Mustard. The role of adhesion in cell cycle progression and drug sensitivity, however, was not addressed in their studies. Importantly, a number of studies analysing sequential tumor biopsy specimens before and after chemotherapy (Briffod et al., 1992; Remvikos et al., 1993; Spyratos et al., 1992) have revealed that responsive tumors show a large G2/M arrest that is almost never observed or that is very minimal in unresponsive tumors when it is observed. A similar association between cell cycle arrest and drug sensitivity has also been noted in human tumor xenografts and experimental animal models using cyclophosphamide and other chemotherapeutic agents (D'Incalci et al., 1983; Ferrari et al., 1989; Jackel and Kopf-Maier, 1991; Wennerberg et al., 1984).

Since cyclophosphamide is known to be preferentially cytotoxic towards rapidly proliferating cells (DeWys, 1972; Tannock, 1978; Braunschweiger and Schiffer, 1978), we reasoned that cell adhesion may regulate or affect cell cycle and in turn may indirectly regulate the drug resistance phenotype. At the molecular level, it is unknown why cyclophosphamide is most active against rapidly dividing cells (DeWys, 1972; Tannock, 1978; Braunschweiger and Schiffer, 1978; Epifanova, 1984). The exact mechanism governing adhesion-dependent resistance in our system also remains unclear, although many possibilities exist. For example, since cell-cell and cell-extracellular
matrix interactions are known to regulate apoptosis, an increase in intercellular adhesion could potentially inhibit cytotoxic drug-induced programmed cell death (Boudreau et al., 1995; Bates et al., 1994; Frisch and Francis, 1994; Meredith et al., 1993) Alternatively, cells in G0/G1 may be less susceptible to DNA damage (Olive et al., 1993; Darzynkiewicz and Traganos, 1982; Rossini et al., 1975; Olive and Durand, 1985) or may be more proficient at repairing their DNA than cells in other phases of the cell cycle (Mendonca et al., 1990; Olive and Durand, 1994; Barcellos-Hoff et al., 1990; Dikomey, 1990). We believe that the observed decrease in growth with increased adhesion is likely to contribute to the acquired or intrinsic resistance of most solid tumors in patients, especially since labelling indexes in such tumors are low, usually ranging between 1 - 15% (Tannock, 1978; Tannock, 1987; Meyer, 1982). Using fine-needle aspirates to collect breast cancer cells from patients, a number of investigators have monitored cell cycle kinetics by flow cytometry before and after chemotherapy (Briffod et al., 1992; Remvikos et al., 1993; Spyros et al., 1992; Osborne, 1989; Remvikos et al., 1989; O'Reilly et al., 1992). Such studies have shown retrospectively that patients with rapidly proliferating tumors responded much better to chemotherapy, at least initially, than those with tumors containing a low S-phase fraction (Briffod et al., 1992; Remvikos et al., 1993; Spyros et al., 1992; Osborne, 1989; Remvikos et al., 1989; O'Reilly et al., 1992; Remvikos et al., 1993; Sulkes et al., 1979; Hietanen et al., 1995). The present study suggests that hyaluronidase may also chemosensitize tumor cells in vivo by stimulating tumor cell growth. Importantly, hyaluronidase treatment by itself did not reduce survival time in vivo.

In summary, this study, which was designed to investigate the nature of multicellular drug resistance, has revealed three novel findings. First, an increase in homotypic cell adhesion, as manifested by an increase in compaction in three-dimensional culture following drug selection in
vivo, is sufficient to confer a high level of resistance to the alkylating agent 4-HC. Second, compact intercellular adhesion represses cycle perturbations following drug exposure, but only when assayed in three-dimensional culture. Thus, compact spheroids mimic what has previously been noted only in resistant versus sensitive tumors of patients or resistant cell lines derived and analyzed in experimental animals, i.e., cells from resistant tumors maintain a large G0/G1 population after drug exposure. Third, hyaluronidase is able to sensitise tumor cells to 4-HC within 24 hours of drug exposure by a mechanism independent of increased drug penetration into the spheroid. This mechanism is likely to be related to the “anti-adhesive” effect of hyaluronidase, which recruits cells into the cycling pool, rendering them more sensitive to 4-HC. Our in vivo results indicate that hyaluronidase is also able to act as a chemosensitizing agent for cyclosphosphamide when both agents are administered simultaneously into mice bearing ascites tumors of EMT-6 compact clones where many of the cells grow as aggregated clusters, i.e. as spheroids. We have also begun to test other cell lines and have observed that hyaluronidase is unable to abolish adhesion and sensitize all tumor cell lines to 4-HC. The chemosensitizing activity of hyaluronidase may depend, therefore, on whether or not HA is cross-bridging cells together. If this is true, in these cases perhaps other tumor specific “anti-adhesives” may be effective in combination with cell-cycle specific drugs (Kerbel et al., 1994; Kerbel et al., 1995). Alternatively, it may be more feasible, at least in the case of solid tumors, to target the downstream signal transduction pathways that are regulated by adhesive interactions at the cell surface.
Chapter 3

Impact of the cyclin-dependent kinase inhibitor p27kip1 on adhesion-dependent resistance of tumor cells to anticancer agents


3.1 Abstract

A low fraction of proliferating cells in solid tumors limits the effectiveness of cell cycle dependent chemotherapeutic agents. To understand the molecular basis of such "kinetic" resistance we cultured tumor cells as multicellular spheroids and examined levels of p27\(^{kip1}\), a cyclin-dependent kinase inhibitor known to be upregulated by intercellular contact in normal cells. When transferred from monolayer to three-dimensional culture, a consistent upregulation (up to 15 fold) of p27 protein was observed in a panel of mouse and human carcinoma cell lines. Antisense-oligonucleotide-mediated downregulation of p27 in EMT-6 mammary tumor cell spheroids reduced intercellular adhesion, increased cell proliferation, sensitized tumor cells to 4-hydroperoxycyclophosphamide, and restored drug- or radiation-induced cell-cycle perturbations repressed in spheroid culture. Our results implicate p27 as a regulator of drug resistance in solid tumors and suggest that tumor-targeted p27 antagonists may be useful chemosensitizers in conjunction with conventional anti-cancer therapy.

3.2 Introduction

The efficacy of current cancer therapy is limited by either intrinsic or acquired resistance to anticancer agents. A major factor contributing to de novo resistance of common solid tumors is a low proliferative fraction (Tannock, 1978; Tannock, 1994). This is important because the majority of anticancer agents in current use are preferentially active against rapidly dividing cells (Tannock, 1978; Tannock, 1994). In some cases, for example, most vinca alkaloids and antimetabolites, the drugs are most active during a particular phase of the cell cycle. In other cases, for example, cyclophosphamide, the drugs do not act in a phase specific manner but are much more effective against rapidly dividing than slowly or nondividing cells. As most solid tumors display a
heterogeneous proliferation rate and contain many quiescent cells, whereas certain normal tissue
types (for example, gut mucosal and bone marrow cells) are mitotically active, the amount of therapy
that can be administered is limited by normal tissue toxicity. Such intrinsic kinetic drug resistance
of tumors can be recapitulated in cell culture by growing tumor cells as multicellular spheroids. In
contrast to monolayer cell cultures, multicellular spheroids display a reduced growth fraction and
resistance to a wide variety of cytotoxic agents (Sutherland, 1988; Olive and Durand, 1994).

Recently, we have shown that the mouse EMT-6 mammary carcinoma cell line, which was
made resistant to alkylating agents in vivo (Teicher, 1990), acquired new in vitro properties that were
detected only when analyzed in spheroid culture (Kobayashi, 1993; St.Croix, 1996a; Kerbel, 1994).
Under these conditions the resistant variants displayed an increase in intercellular adhesion (or
compaction), a decrease in proliferation rate, and manifestation of the in vivo-like drug resistance
phenotype that was lost when cells were grown in monolayer culture. This phenomenon, which we
refer to as acquired "multicellular resistance", may be grossly underappreciated, at least for solid
tumors, due to the almost exclusive use of monolayer culture systems for the study of drug resistance
mechanisms (Hoffman, 1994). Because the drug resistance properties of EMT-6 cells correlated with
a compact morphology, we hypothesised a causal relationship between these two phenotypes.
Adhesive interactions at the cell surface, may be regulating cell proliferation, which in turn may
regulate the level of tumor cell resistance to anticancer agents (St. Croix et al., 1996a). In support
of this view, we also found that addition of the enzyme hyaluronidase simultaneously abolished
intercellular adhesion, stimulated tumor cell proliferation and sensitized EMT-6 tumor cells in vitro
to both an activated form of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC) (St.Croix
et al., 1996a) and γ-irradiation (γ-IR) (unpublished observations). Furthermore, hyaluronidase
sensitized EMT-6 tumor-bearing mice to cyclophosphamide *in vivo* (St.Croix et al., 1996a). In the present study, we were interested in determining the molecular basis of adhesion-dependent (kinetic) resistance to anti-cancer agents.

Progression through the cell cycle is governed by cyclins and their partners the cyclin dependent-kinases (CDKs) (Morgan, 1995). Recently, two families of cyclin dependent kinase inhibitors (CKIs) have been identified which inhibit cell-cycle progression by binding to and inactivating cyclin/CDK complexes (Sherr and Roberts, 1995). The INK family, including p15<sub>INK4b</sub>, p16<sub>INK4a</sub>, p18<sub>INK4c</sub>, and p19<sub>INK4d</sub> bring about G1 arrest by specific inhibition of D-cyclins complexed with cdk4 or cdk6. In tumor cells mutations are prevalent in p15 and p16 suggesting that they may act normally as tumor suppressor genes (Hunter and Pines, 1994). The p21 family of CKIs includes p21<sup>Waf1.Cip1.Sdi1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>. Although p21 and p27 can inhibit many cyclin/CDK complexes *in vitro*, their *in vivo* activity appears to be restricted to the G1 cyclin/CDK complexes (Sherr and Roberts, 1995). Although genetic alterations are common in INK family members, mutations in p21 and p27 appear to be rare in tumors (Ponce-Castaneda et al., 1995; Kawamata et al., 1995; Shiohara et al., 1994 Ferrando et al., 1996; Spirin et al., 1996), suggesting that tumor cells retaining such inhibitors may have a survival advantage. This finding, coupled with the observation that p27 is upregulated by cell-cell contact in normal cells in culture (Slingerland et al., 1994; Hengst et al., 1994; Polyak et al., 1994), stimulated us to examine expression of p27 in tumor cells growing in a three-dimensional context. Our results implicate p27 as an important regulator of the adhesion-dependent resistance of tumor cells to anticancer therapy.
3.3 Materials and Methods

3.3.1 Cell lines and culture conditions

The human MCF-7 breast carcinoma cell line, the murine EMT-6 mammary carcinoma (EMT-6/P) cell line and its in vivo derived alkylating-agent resistant variants E/CTX and E/DDP (Teicher, 1990) were a gift from Beverly Teicher (Dana-Farber Cancer Institute, Boston, MA). E/Pc5-T, E/Pc7-T and E/Pc10-L are "tightly" (T) or "loosely" (L) adherent clones of the EMT-6/P cell line which were described previously (St.Croix, 1996a). All EMT-6 cells were cultured in Waymouth's MB 752/1 medium, supplemented with 10% FBS. For hyaluronidase treatment of E/Pc5-T cells, 2mg/ml of bovine testicular hyaluronidase (Sigma) was added to the culture medium at the time of cell plating, and cells were collected and assayed three days later. The human MDA 435 breast carcinoma cell line and its paclitaxel-resistant variant 435/TO.3, a gift from Dalia Cohen (Sandoz Research Institute, East Hanover, NJ), were grown in DMEM supplemented with 10% FBS, 0.1mg/ml sodium pyruvate and 0.1mM nonessential amino acids. The human ovarian A2780 carcinoma and its cisplatin-resistant variant A2780/PDD, donated by Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA) were grown in RPMI 1640 supplemented with 10% FBS. All other cell lines were purchased from American Type Culture Collection [(ATCC), Rockville, MD] and were grown in DMEM with 10% FBS except BT549 and HBL100 which were grown according to ATCC's recommended protocol. Multicellular aggregates were prepared by using the liquid overlay method as previously described (Kobayashi et al., 1993). Briefly, SeaPlaque agarose (FMC Bioproducts, Rockland, ME) was diluted to 1% with serum-free medium and coated (0.25 ml) into each well of 24-well plates. Tumor cells (10^5 in 1 ml of complete medium) were then plated on top of the solidified agarose.
3.3.2 Immunoblotting

Cells from monolayer culture were harvested in exponential growth phase, whereas three-dimensional cultures (St.Croix, 1996a) were grown for three days before harvesting unless otherwise stated. Cells were treated for 3 days with hyaluronidase and 24 hours with 6 μM 4-HC. After the cells were collected, they were rinsed with PBS and stored as cell pellets at -70°C until ready for use. Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20mM Tris-HCl pH7.5, 137mM NaCl, 100mM NaF, 1mM sodium vanadate, 1mM phenylmethyl sulphonyl fluoride and 0.02mg/ml each of aprotinin, leupepsin and pepstatin). The lysates were sonicated and clarified by centrifugation, resolved by SDS polyacrylamide gel electrophoresis, blotted onto Immobilon-P-membranes (Millipore Corporation, Bedford, MA) and probed with a mouse monoclonal p27 antibody (Transduction Laboratories, Lexington, KY) diluted 1:1000 or a rabbit polyclonal p21 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in TBST (Tris Buffered Saline-0.25% Tween 20) containing 5% dry milk. After washing in TBST, the immunoreactive proteins were visualized using horseradish peroxidase-conjugated antimouse-IgG (Promega Corporation, Madison, WI) diluted 1:5000 and the enhanced chemiluminescence (ECL) western blotting detection system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

3.3.3 Immunohistochemical staining of p27

E/Pc5-T spheroids grown in three-dimensional culture for 3 days were placed into a 1.5 ml Eppendorf tube, spun for 5 min (800 r.p.m.), rinsed with PBS, and fixed in 10% formalin. Paraffin-embedded specimens were sectioned, deparaffinized with xylenes, rehydrated and microwaved for 10 minutes in citrate buffer (pH 6.0). Sections were blocked for endogenous peroxidase with 3%
hydrogen peroxide in methanol then blocked for non-specific staining with immunoglobulins from normal horse serum (1:20 dilution). After removing excess blocking serum, sections were incubated overnight with monoclonal p27 antibody diluted 1:1000 (0.25μg/ml) in PBS, followed by incubation with biotin-labelled anti-mouse secondary antibody. The sections were incubated with preformed avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Metal enhanced diaminobenzidine (DAB) substrate (Pierce, Rockford, IL) was then added in the presence of Horseradish Peroxidase. Sections were counterstained with hematoxylin, dehydrated and mounted with permount (Fisher Scientific, Nepean, ON).

3.3.4 Oligonucleotide treatment

The sequences of the antisense (AS) and mismatch (MSM) p27 C-5-propyne modified phosphorothioates utilized in the experiments (designated AS1 and MSM1 in the [3H]-thymidine incorporation assay) were 5'-GCGUCUGCUCCACAG-3' and 5'-GCAUCCCCUGUGCAG-3', respectively. The alternative oligonucleotides labelled AS2 and MSM2 in the [3H]-thymidine assay were 5'-UGGCUCUCCUGCAGGC-3' and 5'-UCCCUUUGGCGCGC-3', respectively. For maximum and uniform delivery, all oligonucleotide treatments were performed on rapidly growing monolayer cultures. For transfection, C5-propyne-modified oligonucleotides (Gilead Sciences, Foster City, CA) at 20X the final concentration in EX-CELL-300 medium (pH7.2, JRH Biosciences, Lenexa, Kansas) were heated (65°C) for 5 minutes to denature secondary structure, and then mixed at room temperature with a 20X solution of GS2888 cytofectin (Gilead Sciences). After 10-15 minutes, oligo/cytofectin complexes were diluted to 1X concentration in EX-CELL-300 medium and overlaid onto cells. Final concentrations used were 2μg/ml for GS2888 and 5-60nM for
oligonucleotides. After 5 hours of incubation with oligonucleotides, cells were rinsed with PBS, harvested with trypsin, and then plated into various assays in complete medium containing 10% FBS.

3.3.4 Proliferation assay

E/Pc5-T cells treated with antisense or mismatch oligonucleotides in monolayer culture were harvested, and 5000 cells/well in 100μl of complete medium were added to 96-well U-bottom plates (Nunc). After 48 hours of incubation, 2μCi of [3H]-thymidine was added in 50μl to each well and plates were pulsed for 4 hours. Labelled cells were frozen at -70°C and later harvested onto filtermats using a Titertek cell harvester 530. Radioactive filtermats were then counted using a 1205 beta plate liquid scintillation counter (Fisher Scientific). The rate of DNA synthesis of the antisense- or mismatch-treated groups was calculated as a fraction of the cytofectin-treated controls. To prevent attachment of cells to the bottom of 96-well plates, a heated (56°C) solution of 2% poly(2-hydroxyethylmethacrylate) (polyhema) (Aldrich Chemical Company, Inc. Milwaukee WI) in ethanol was briefly added to and then removed from the plate leaving behind a thin film in each well.

3.3.5 Monitoring cell cycle changes following exposure to 4-HC or γ-IR

Antisense- or mismatch-treated cells from monolayer culture were harvested and plated into three-dimensional culture (Kobayashi, 1993). After 48 hours of growth in suspension, cells were treated with either 20μM 4-HC or 20Gy γ-IR and left for an additional 48 hours before collection. For DNA analysis, cells were disaggregated with trypsin, rinsed with PBS, fixed in 70% ethanol, and stained with PI solution (50μg/ml propidium iodide, 10μg/ml RNase A in PBS). Cells were
analyzed using the Lysis II software on a FACScan flow cytometer (Beckton-Dickinson, San Francisco, CA). Cell-cycle phase distributions were calculated using Cell Fit software (Becton Dickinson).

3.3.6 Tumorigenicity assay

E/Pc5-T cells treated with oligonucleotides in monolayer culture were harvested and plated into three-dimensional culture. Two days later, tumor cells were exposed to 10 or 15μM 4-HC and after a further 24 hours, rinsed with PBS, trypsinized and 5x10^5 cells were injected subcutaneously into syngeneic BALB/c mice. Tumor volume (V) was calculated using the formula \( V = \frac{d^2D}{2} \) where \( d \) is the smallest tumor diameter (mm) and \( D \) is the largest tumor diameter (mm).

3.4 Results

3.4.1 Overexpression of p27 in aggregated tumor cells

Our previous studies have suggested that an acquired adhesion-dependent decrease in cell proliferation may be necessary for the resistance of tumor cells to cytotoxic agents (St.Croix, 1996a). To understand the molecular basis of this phenomenon, we analyzed levels of p21 and p27 in our clonally derived variants of the EMT-6 mammary carcinoma cell line. The two variants used, E/Pc10-L and E/Pc5-T, were clones that spontaneously formed either loosely or tightly adherent aggregates, respectively, when grown in three-dimensional culture (St.Croix, 1996a). We have also taken advantage of the fact that compact intercellular adhesion can be disrupted in E/Pc5-T cells by treatment with hyaluronidase.

p21 and p27 protein levels were relatively constant in both tight and loose clones grown as
asynchronous monolayer cultures, with or without the addition of hyaluronidase (Fig. 3.1A). In contrast, an increase in the level of p27 was observed in three-dimensional culture. This was most pronounced in cells of the tightly adherent clone, E/Pc5-T (approximately 10- to 12-fold increase in p27 protein). In contrast, the loosely adherent E/Pc10-L clone and the hyaluronidase treated E/Pc5-T clone, displayed a more moderate increase (6- to 7-fold) when transferred from two- to three-dimensional culture. In E/Pc5-T cells the increase in p27 expression in three-dimensional culture was gradual and paralleled an increase in aggregate compaction. After 8 hours in suspension, p27 levels were unchanged, but by 24 hours an increase in p27 was apparent (Fig. 3.1B). The level of p27 continued to increase throughout the 96 hour time course. In contrast, p27 expression in hyaluronidase-treated cells increased rather slowly and never reached the same level as in adherent E/Pc5-T cells (data not shown; see also figure 3.1A). Unlike p27 levels, p21 levels were relatively high in monolayer and were undetectable by 96 hours in cells grown as aggregates (fig. 3.1B) Thus, increased levels of p27 correlated with increased cellular compactness in three-dimensional culture.

We analyzed p21 and p27 protein levels 24 hours after exposure to a low concentration (6μM) of 4-HC (Fig. 3.1A). In rapidly proliferating monolayer cultures, a decrease in p27 level was evident following drug exposure. This decrease coincided with a large G2/M arrest (see figure 2.4, chapter 2). In three-dimensional culture, where the G2/M arrest is attenuated, p27 levels were relatively unaffected by exposure to 4-HC.

We also examined the expression of p27 in a panel of human breast, colon and ovarian cancer cell lines after plating cells into suspension (fig. 3.2A). In every cell line tested, we observed an increase in p27 levels (~1.2- to 15-fold) in three-dimensional culture. Again, no such correlation was
Figure 3.1 A) Increase in p27 but not p21 protein levels in adherent EMT-6 cells grown as three-dimensional aggregates. Western blot of E/Pc5-T (tight) and E/Pc10-L (loose) cells collected from either exponentially growing monolayer or 3-day-old three-dimensional cultures. Hyaluronidase (HYase) treatment disrupted intercellular adhesion of E/Pc5-T cells under these conditions, converting them to a "loose" E/Pc10-L-like morphology. Compact spheroids in three-dimensional culture consistently displayed higher levels of p27 but not p21 compared with loose aggregates and monolayer cultures. Treatment with 6μM 4-HC led to a decrease in p27 but not p21 in monolayer, but not in three-dimensional culture.
Figure 3.1 B) Time course of changes in p27 and p21 levels after transferring the cells into three-dimensional culture. E/Pc5-T cells were grown for the indicated times in three-dimensional culture after which p27 and p21 protein levels were analysed by immunoblotting. Control cells (time 0) represent monolayer cultures.
Dimensional culture, whereas the effect on p21 varied between cell lines.

p27 protein levels by immunoblotting. Note that p27 protein levels are consistently elevated in three-

549, MDA468, HBL100, and colon (SW480, H.T29) carcinoma cell lines were probed for p21 and

luminal breast (MCF-7, BT-

lure cell lines in monolayer (2D) or three-dimensional (3D) culture. Human breast (MCF-7, BT-

Figure 3.2 A) p21 and p27 protein levels in various mouse and human breast, colon and ovarian

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noted for p21 expression except in the case of the SW480.7 colon carcinoma.

We also undertook immunohistochemistry on cross-sections of E/Pc5-T spheroids to determine the pattern of p27 staining. Intense nuclear staining of p27 was found in cells distributed throughout the spheroid (Fig. 3.3). Only a few unstained cells were observed, and these were found either in the outermost rim of proliferating cells or in the centre of the spheroid near the necrotic core. The layer of tightly packed cells directly beneath the outermost proliferating rim stained most intensely. As expected, hyaluronidase-treated aggregates displayed reduced nuclear staining (data not shown). This pattern is consistent with a role for intercellular adhesion, in regulating p27 production.

3.4.2 p27 levels elevated in drug resistant cell lines

We compared the levels of p21 and p27 in the MDA 435 breast cancer cell line and its in vitro derived paclitaxel-resistant variant, 435/TO.3. Little p27 was noted in monolayer culture, whereas in three-dimensional culture, both lines showed an increase in p27. In three-dimensional culture p27 expression was much higher in 435/TO.3 than in parental MDA 435 cells. The same pattern was also observed for the human ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780/PDD. The in vivo derived cyclophosphamide resistant variant of the EMT-6/P cell line, E/CTX, displayed more p27 than the parental cell line, but only in three-dimensional culture. The cisplatin resistant variant, however, displayed higher p27 levels than EMT-6/P in both two- and three-dimensional culture. Notably, all of these drug resistant variants also displayed an obvious increase in intercellular adhesion as multicellular aggregates compared to their looser parental counterparts (Kobayashi, 1993) (unpublished observations).

An increase in p21 was evident only in two human drug-resistant variants relative to their
Figure 3.2 B) p27 and p21 protein levels in drug resistant cell lines. Western blot of breast (EMT-6, MDA 435) and ovarian (A2780) cell lines, and their drug-selected variants (E/CTX, E/DDP, 435/TO.3, A2780/PDD). Protein was extracted from subconfluent monolayer cultures (2-D) or three day old three-dimensional cultures (3-D). Note that p27 protein levels are consistently elevated in the drug-resistant variants compared with their parental counterparts in three-dimensional culture.
Figure 3.3 Intense p27 immunostaining throughout cross-sections of E/Pc5-T spheroids. After 3 days of growth in three-dimensional culture spheroids were collected and embedded in paraffin, and cross-sections were stained using a p27 monoclonal antibody. Note the prominent nuclear staining in the majority of cells and the presence of a few unlabelled cells in the outer rim and near the necrotic core. Mitotic cells were not stained.
respective parental cell lines (fig. 3.2B). Thus, in three-dimensional culture acquisition of a drug resistance phenotype correlated with a consistent increase in intercellular adhesion and the level of p27, but not p21.

3.4.3 Impact of p27 on adhesion and proliferation

To determine whether an increase in p27 was responsible for the decreased growth kinetics of these cells in three-dimensional culture, we employed a new generation of oligonucleotides which contain C5-propyne-modified bases (Wagner et al., 1993) to downregulate p27. We used two different 15-base antisense oligonucleotides and two different mismatch control oligonucleotides with the same base composition but with a partially scrambled sequence. These same oligonucleotides have previously been shown to be specific for p27 by a study exploiting the degeneracy of the genetic code to construct a p27 expression plasmid that could not be inhibited by the antisense oligonucleotides but nevertheless encoded wild-type p27 protein (Coats et al., 1996). We have observed efficient delivery of oligonucleotides to the cells (90-95% in the nucleus) by using the cationic lipid GS2888 cytofectin and fluorescein isothiocyanate (FITC)-labelled oligonucleotides (data not shown). Antisense treatment led to a dose-dependent specific reduction in the level of p27 protein (by a factor of 2.5) most notable after 48 hours (Fig. 3.4A). Our finding that this reduction in p27 protein level caused profound alterations in the cell cycle profile (see below) is consistent with the observation of Reyniskottir et al. that a 2- to 3-fold induction of p27 was sufficient to saturate cyclinE/cdk2 (Reyniskottir et al., 1995). The expression of p21 protein was unaltered by the antisense oligonucleotides (data not shown).

Pre-treatment with antisense p27 oligonucleotides resulted in abrogation of tight intercellular
Figure 3.4 A) Antisense oligonucleotides against p27 stimulate growth of E/Pc5-T cells. Cells treated with 7.5-60 nM of either antisense or mismatch oligodeoxynucleotides (ODNs) in monolayer culture were placed into three-dimensional culture in complete medium and 48 hours later pulsed with $[^3H]$-thymidine. The result shown is representative of at least three independent experiments.
aggregation of E/Pc5-T cells in three-dimensional culture (Fig. 3.5). This morphological change appeared to be specific, as it occurred with two independent antisense oligonucleotides but not with two mismatch controls (data not shown). As shown in Fig. 3.4B, the same two independent antisense oligonucleotides caused a marked dose-dependent increase in $[^3$H]-thymidine incorporation. The two control mismatch sequences had a negligible effect at low concentrations (8-20nM) but caused a slight increase in $[^3$H]-thymidine uptake at higher (30-60nM) concentrations. The effect of antisense p27 on both DNA synthesis and adhesion was transient reaching a maximum at about 48 hours.

3.4.4 Impact of p27 on cell cycle kinetics and 4-HC resistance

We observed previously that loose aggregates of hyaluronidase-treated E/Pc5-T cells arrested in G2/M after exposure to 4-HC (St.Croix, 1996a) or γ-IR (unpublished observations) in three-dimensional culture. In contrast, if hyaluronidase was absent the G2/M arrest was almost completely attenuated in E/Pc5-T tight spheroids following exposure to these agents. To determine whether the level of p27 affected these cell cycle perturbations, we treated E/Pc5-T cells with antisense or mismatch p27 oligonucleotides and then evaluated cells by flow cytometry for drug- or radiation-induced cell-cycle changes (Fig. 3.6). Forty-eight hours after treatment with antisense but not mismatch p27 oligonucleotides, we observed recruitment of cells into the cell cycle (44.5% vs 28.4% S phase, see figure 6). This effect disappeared by 96 hours (data not shown). After exposure to 4-HC for 48 hours, a much larger proportion of cells were arrested in G2/M in the antisense-treated group than in the mismatch controls. A similar pattern was also observed following exposure to γ-IR.
Antisense oligonucleotides inhibit p27 protein expression of E/Pc5-T cells. Cells treated with 5-30 nM of antisense (AS-p27) or mismatch (MSM-p27) oligonucleotides were collected for western blot analysis after 48 hours of growth in three-dimensional culture. Antisense-treated cells showed a dose-dependent decrease in p27 expression. The result shown is representative of three independent experiments.
Figure 3.5  p27 antisense oligonucleotides abolish tight intercellular adhesion of E/Pe5-T cells.

Cells growing for 48 hours on either polyHEMA coated U-bottom 96-well plates (top) or six-well tissue culture plates coated with agarose (bottom) were photographed under X40 magnification. Even when forced together in the U-bottom wells, antisense-treated cells did not compact into a spheroid but instead formed a wide, loose sheet of cells, 5-10 cell layers thick. (Bar = 0.5mm).
Figure 3.6 Effect of antisense p27 oligonucleotides on cell-cycle distribution after exposure to γ-IR and 4-HC. E/Pc5-T cells treated with antisense or mismatch p27 oligonucleotides were grown in three-dimensional culture for 48 hours, exposed to 20Gy γ-IR or 20μM 4-HC, and 48 hours later, cells were collected and DNA profiles were analyzed by flow cytometry.
When E/Pc5-T cells, treated with oligonucleotides, were placed back into monolayer culture instead of spheroid culture, no difference in cell-cycle distribution was observed in cells treated with antisense or mismatch oligonucleotides alone (54.9% and 52.6% of cells in S phase, respectively). After exposure to either 4-HC or γ-IR, a majority of the cells in monolayer culture were arrested in G2/M with no obvious difference between the antisense- and mismatch-treated groups (data not shown).

To evaluate the role of p27 in drug resistance, we employed a tumorigenicity assay (St.Croix, 1996a). E/Pc5-T tumor cells treated with oligonucleotides were plated into three-dimensional culture and 48 hours later exposed to 4-HC. After a further 24 hours, cells were harvested and injected subcutaneously into syngeneic BALB/c mice. When cells were treated with antisense or mismatch oligonucleotides alone, no difference in tumor growth was noted. However, when 4-HC was added to the medium, obvious differences in tumorigenicity became apparent (see Figure 3.7). When exposed to 10 or 15μM 4-HC, antisense treatment led to a significantly longer in vivo latency period than in the mismatch-treated controls. Furthermore, at the higher drug concentration, tumor take was lower in the antisense-treated than in the mismatch-treated cells. Control cells without oligonucleotide-treatment behaved similarly to mismatch-treated cells (data not shown). Thus, antisense treatment resulted in greater tumor cell kill by 4-HC.

3.5 Discussion

Recruitment of cells into the cell cycle has been shown to enhance tumor cell kill by the use of hyperfractionated radiotherapy schedules or the combined administration of growth stimulatory hormones or cytokines with chemotherapeutic agents. However, effects on patient survival have
Figure 3.7 Tumorigenicity assay of antisense or mismatch p27-treated E/Pc5-T cells after exposure to 4-HC. After 2 days of growth in three-dimensional culture, oligonucleotide-treated cells were exposed to 10 or 15μM 4-HC, and 24 hours later 5 x 10^5 cells were injected into BALB/c mice. Tumor volume, average ± s.e.m.. Tumor take is indicated in the legend.
been variable (Conte et al., 1987; Gore et al., 1995). A better understanding of the molecular machinery regulating cancer cell growth may offer new avenues for therapeutic intervention (Karp and Broder, 1995) and could potentially be exploited to overcome problems encountered with conventional cell-cycle recruitment strategies.

3.5.1 Expression of \( p27 \) is regulated by cellular topology

A solid tumor mass, by definition, contains cells in close proximity to one another. Levels and/or activity of \( p27 \) is known to be upregulated in normal cells by cell-cell contact (Slingerland et al., 1994; Hengst et al., 1994; Polyak et al., 1994). Our studies demonstrate that this is also true in tumor cells. The observed increase in the level of \( p27 \) in three-dimensional culture appears to be regulated by at least two different factors. First, even cells that formed loosely adherent aggregates (for example, loose clones, or hyaluronidase-disrupted tight clones) showed a 6- to 7-fold increase in \( p27 \) levels over monolayer cultures. This is consistent with a recent report showing a similar increase in \( p27 \) (~4-fold) in both normal and transformed fibroblasts in suspension culture (Fang et al., 1996). The signals regulating this increase in \( p27 \) could be related to a change in cell shape or a change in the cells ability to adhere to and receive signals from an extracellular matrix. The second factor upregulating \( p27 \) levels appears to be the degree of intercellular adhesion. This was shown in both our E/Pc5-T cell line and in our drug-selected tumor cell variants, which all display a tight spheroid morphology and even higher levels (up to 12-fold) of \( p27 \) than their respective loosely adherent counterparts. Taken together, these results suggest \( p27 \) levels in tumor cells can be strongly upregulated by multiple factors including intercellular adhesion. It is noteworthy that depletion of \( p27 \) from tumor cells resulted in reduced intercellular adhesion suggesting that the
reverse is also true, that is, that intercellular adhesion is also under control of the cell cycle machinery.

3.5.2 The relationship between p27 expression, cell-cycle kinetics and tumor cell death induced by DNA damaging agents

To determine the role of p27 in adhesion-dependent growth regulation and drug resistance, an antisense approach was employed to deplete p27 from tumor cells. For this purpose we utilized a new generation of oligonucleotides which contain C5-propyne-substituted bases (Wagner, 1993). Due to an enhanced affinity for complementary RNA, lower concentrations of these modified oligonucleotides can be used making them potentially more specific than previously used phosphorothioates. Indeed, an elegant study by Roberts and co-workers has proven the specificity of the same set of p27 oligonucleotides that we employed. They achieved this by exploiting the degeneracy of the genetic code to construct a p27 expression plasmid that was unaffected by antisense oligonucleotides but nevertheless encoded wild-type p27 protein (Coats et al., 1996).

Cells in compact spheroids expressing high p27 levels fail to arrest significantly in G2/M after exposure to 4-HC or γ-IR. By increasing the proliferative fraction, antisense but not mismatch oligonucleotides to p27 sensitized cells to DNA damaging agents, with resultant arrest in G2/M. This attenuated G2/M arrest appears to be specific to tightly adherent cells growing in a three-dimensional context (St.Croix, 1996a; Sano et al., 1983; Hinz and Dertinger, 1983). As the G2/M arrest is predictive of drug sensitivity (St.Croix, 1996a) and has also been correlated with tumor response to chemotherapy in breast cancer patients (Remvikos et al., 1993; Briffod et al., 1992; Spyrotes et al., 1992), monitoring cytotoxic drug-induced cell-cycle alterations and p27 levels in
tumor cells in three-dimensional culture may be of greater importance than has heretofore been appreciated.

It is unclear why a p27-dependent accumulation of cells in G0/G1 is associated with resistance to DNA damaging agents. One potential explanation is that tumor cells are protected from drug induced apoptosis by cell-cell or cell-extracellular matrix interactions in compact spheroids (Meredith et al., 1993; Frisch and Francis, 1994; Bates et al., 1994) or by high levels of p27. Another possibility is that G0/G1 cells have an enhanced capacity to repair DNA lesions through a process known as potentially lethal damage repair (PLDR) (Mendonca et al., 1990; Little et al., 1973). Adherent, slowly proliferating spheroid cells may also be less susceptible to DNA damage due to altered DNA "packaging" (Olive and Durand, 1994). Increased p27 levels may also help to explain the so-called "contact effect" (Sutherland, 1988; Olive and Durand, 1994) or "confluence-dependent resistance" (Dimanche-Boitrel et al., 1993; Dimanche-Boitrel et al., 1992), two terms used to describe the relative resistance of spheroids or confluent monolayer cultures to a variety of cytotoxic agents.

### 3.5.3 Targeting p27 as a potential strategy to augment anticancer therapy

Our antisense results demonstrate that p27 is essential for the adhesion-dependent resistance of solid tumor cells to DNA-damaging agents. One recent report suggests that an increase in p21 is also associated with chemoresistance in patients with acute myelogenous leukemia (Zhang et al., 1995). In this sense p27 and p21 (Zhang et al., 1995; Poluha et al., 1996; Waldman et al., 1996) may be viewed as survival genes. Thus, downregulation of p27 and perhaps p21 in some tumor types, represents a new potential strategy for augmenting anticancer therapy. Of concern with any new
strategy, however, is the potential for a simultaneous increase in normal tissue toxicity. If normal tissues retain intact CKIs such as p15 and p16, these inhibitors may be able to compensate for the loss of a single CKI such as p27, thus minimizing toxicity. Therefore, an enhanced therapeutic index may depend on tumor cells having lost some CKIs, such as the INK family members, but retaining others, such as p21 and p27. It is noteworthy that p21 and p27 knockout mice are viable, do not manifest any gross morphological abnormalities and, with the exception of benign pituitary adenomas (reported only in the p27 homozygous knockout mice), do not appear to have an increased propensity for developing tumors (Deng et al., 1995; Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). The present results suggest that the anti-adhesive agent hyaluronidase may also be exerting its chemosensitizing effects on EMT-6 cells in vivo (St.Croix, 1996a) indirectly, by downregulating p27. Specific targeting of p27 through an antisense approach in vivo (Tonkinson and Stein, 1996), or through other low molecular weight pharmacological inhibitors of p27, may also prove effective.

With respect to the clinical relevance and application of our results, a series of reports relating cell-cycle parameters with drug or radiation sensitivity (primarily in breast cancer patients), have shown that tumors with a high S-phase fraction almost always respond better, at least initially, than those with slower growing tumors (Remvikos et al., 1993; Spyrouos et al., 1992; Hietanen et al., 1995; O'Reilly et al., 1992; Remvikos et al., 1989). Moreover, the tumor types that are considered curable by chemotherapy, such as childhood tumors, some lymphomas, choriocarcinoma and testicular carcinoma, tend to grow very rapidly (Tannock, 1978; Tannock, 1994).

In summary, our studies demonstrate that adherent tumor cells generally express relatively high levels of p27, but not necessarily p21, in three-dimensional culture. This may help to explain why
most solid tumor types, despite harbouring multiple oncogenes and tumor suppressor genes, often contain a low growth fraction in vivo (Tannock, 1978; Tannock, 1994). Furthermore, cell lines with acquired resistance to chemotherapeutic agents show an increase in both intercellular adhesion and p27 levels, but again only when grown as three-dimensional aggregates. Enforced p27 downregulation resulted in decreased intercellular adhesion, increased cell growth and altered cell cycle kinetics following drug and radiation treatment. Downregulation of p27 also sensitized tumor cells to 4-HC, implicating p27 as a potential mediator of both acquired and intrinsic resistance to certain anticancer agents. Thus CKI antagonists represent a potential novel class of chemosensitizers in the rational treatment of solid tumors with anticancer agents.
Chapter 4

E-cadherin-dependent growth suppression is linked to overexpression of the cyclin-dependent kinase inhibitor p27\(^{KIP1}\)


Submitted: *J. Cell Biol.*
4.1 Abstract

Recent studies have demonstrated the importance of E-cadherin, a homophilic cell-cell adhesion molecule, in contact inhibition of growth of normal epithelial cells. Some types of tumor cells also maintain strong intercellular adhesion and are growth inhibited by cell-cell contact, especially when grown in three-dimensional culture (St.Croix et al., 1996b). To determine if E-cadherin could mediate contact-dependent growth inhibition of non-adherent EMT-6 mouse mammary carcinoma cells which lack E-cadherin, we transfected these cells with an exogenous E-cadherin expression vector. E-cadherin expression in EMT-6 cells resulted in multicellular spheroid formation and a reduced proliferative fraction in three-dimensional culture. In addition to increased cell-cell adhesion, E-cadherin expression also resulted in dephosphorylation of the retinoblastoma protein (pRb), a reduction in cyclin D1 and an increase in the level of the cyclin-dependent kinase inhibitor, p27(kip1). In tightly adherent spheroids we also found increased levels of p27 bound to the cyclin E-cdk2 complex, and a reduction in cyclin E-associated cdk2 kinase activity. Exposure to E-cadherin neutralizing antibodies in three-dimensional culture simultaneously prevented adhesion and stimulated proliferation of E-cadherin transfectants as well as a panel of human colon, breast and lung carcinoma cell lines which express functional E-cadherin. To test the importance of p27 in E-cadherin dependent growth inhibition, we engineered E-cadherin positive cells to express inducible p27. By forcing the expression of p27 levels similar to those observed in aggregated cells the stimulatory effect of E-cadherin neutralizing antibodies on proliferation could be counteracted. This study demonstrates that E-cadherin, classically described as an invasion suppressor, is also a major growth suppressor especially under multicellular growth conditions, and its ability to suppress growth is mediated by increased levels of the cyclin-dependent kinase inhibitor p27.
4.2 Introduction

Intercellular adhesion molecules and extracellular matrix (ECM), once viewed simply as a "cellular glue" and mechanical scaffolding, are now known to have profound affects on cell behaviour (Juliano and Haskill, 1993). For example, integrin mediated binding of normal cells to ECM is known to promote the growth and survival of many cell lines in culture (Bottazzi and Assoian, 1997; Meredith and Schwartz, 1997). As well, recent studies suggest that intercellular adhesion molecules are necessary for non-transformed cells growing in monolayer tissue culture to become quiescent when they reach confluence by a process known as "contact inhibition" (Aoki et al., 1991; Kandikonda et al., 1996; Takahashi and Suzuki, 1996). Although much has been learned recently about the growth promoting effects of cell-ECM interactions (Assoian, 1997; Bottazzi and Assoian, 1997), much less is known about how cell-cell contact inhibits cell cycle progression.

Many tumor cell lines are subject to contact-dependent growth inhibition, although not to the same degree as normal cells. Because solid tumors usually grow as three-dimensional masses where cell-cell contacts are maintained, we decided to study cells cultured as aggregates in suspension. Under such conditions, we observed an even greater degree of contact-dependent growth inhibition than was observed in confluent versus subconfluent monolayer cultures. In clonal ly-derived variants of the EMT/6 mouse mammary carcinoma cell line, for example, we were able to show that the rate of proliferation in three-dimensional culture depended largely on the degree of intercellular adhesion, since loosely-adherent variant cell lines grew significantly faster than tightly-adherent cell lines (St.Croix et al., 1996b). Although the precise mechanism controlling intercellular adhesion in tightly-adherent EMT/6 variant cell lines is as yet unknown, addition of hyaluronidase could abolish such adhesion; we therefore refer to this adhesion mechanism as Hyaluronic Acid (HA)-dependent.
Furthermore, tightly-adherent variants dispersed with hyaluronidase grew at a rate similar to that observed for loosely-adherent EMT/6 variants.

The recent identification of several key regulators of cell cycle progression provides a valuable new avenue for delineating the molecular mechanisms underlying contact inhibition. The cell cycle is governed by the sequential activation and inactivation of a family of cyclin-dependent kinases (CDKs) (Morgan, 1995). Cdk activation requires cyclin association and cyclin levels typically oscillate throughout the cell cycle. Because contact inhibited cells enter quiescence or the G1/G0 phase of the cell cycle, molecules regulating G0/G1-phase are likely to be particularly important in understanding contact inhibition. Progression through G1 into S-phase is regulated by cyclin D associated with Cdk4 or Cdk6 and by cyclin E/cdk2. Subsequently, as cells enter S phase, cyclin A/cdk2 is activated. Recently, two families of cyclin-dependent kinase inhibitors (CKIs) have been identified which bind to and inhibit the activity of cdks (Sherr and Roberts, 1995). The INK family is comprised of p15INK4B, p16INK4A, p18INK4C and p19INK4D. INK family members share four ankyrin motifs and inhibit specifically cyclin D-dependent cdks (cdk4 and cdk6). Three Cip/Kip family members have also been identified and include p21Cip1/WAF1, p27Kip1 and p57Kip2. Members of this family show broader substrate specificity and inhibit the activity of both cyclin D-cdk4/6 and cyclin E-cdk2. Because levels and/or activity of p27 are elevated upon cell-cell contact of non-transformed fibroblasts or epithelial cells (Hengst et al., 1994; Kato et al., 1997; Polyak et al., 1994; Slingerland et al., 1994; Winston et al., 1996), this inhibitor may be of particular importance in the regulation of contact inhibition. Similarly, our recent studies demonstrate that p27 levels are increased in many breast, colon and ovarian carcinoma cell lines when transferred from two- to three-dimensional culture. Importantly, depleting p27 from tumor cells using an antisense strategy
resulted in a significant growth stimulation over controls, but only in three-dimensional culture (St. Croix et al., 1996a). Thus, p27 is at least in part responsible for contact-dependent growth inhibition of carcinoma cells. However, the fact that normal fibroblasts from p27 -/- mice can undergo contact inhibition (Nakayama et al., 1996) suggests that there may be cell type specificity or redundancy in the regulation of contact inhibition at the molecular level.

The role of cell adhesion molecules in contact-dependent signalling pathways which ultimately affect p27 levels is unclear. In this study, we investigated the effect of E-cadherin mediated intercellular adhesion on both proliferation and p27 levels. E-cadherin is a major calcium-dependent homophilic cell adhesion molecule found on normal epithelial cells. Its intracellular domain binds directly to β-catenin which, along with α-catenin, links E-cadherin to the underlying actin cytoskeleton (Aberle et al., 1996). In addition to its role in adhesion, β-catenin has also been implicated in Wnt signal transduction and interacts with the APC tumor suppressor protein as well as transcription factors of the LEF/TCF family (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Rubinfeld et al., 1993; Su et al., 1993). By sequestering β-catenin at the cell surface, cadherins have been shown to antagonize β-catenin signalling pathways which are required for normal dorsoventral axis formation in *xenopus* (Fagotto et al., 1996). Carcinomas in which E-cadherin expression is lost or reduced (Becker et al., 1994; Berx et al., 1996), may acquire a relative growth advantage over normal contact-inhibited cells. Indeed, a few studies have suggested a potential role for E-cadherin as a growth suppressor (Hermiston and Gordon, 1995b; Kandikonda et al., 1996; Miyaki et al., 1995; Navarro et al., 1991; Takahashi and Suzuki, 1996; Watabe et al., 1994). However, the mechanism(s) by which E-cadherin affects the cell cycle machinery have not been elucidated.
In the present study, we investigated the roles of E-cadherin and p27 in contact-dependent growth inhibition of carcinoma cells by engineering EMT-6 tumor cells which are poorly adherent in three-dimensional culture to express functional E-cadherin, and by antagonizing E-cadherin mediated adhesion using anti-E-cadherin neutralizing antibodies. Carcinoma cells which maintain E-cadherin function were significantly inhibited by cell-cell contact when grown in a three-dimensional context. Importantly, E-cadherin-dependent growth inhibition was associated with increased levels of the cyclin-dependent kinase inhibitor p27. One mechanism by which E-cadherin can elevate p27 is through inhibition of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR). Our results suggest that E-cadherin is not only an "invasion suppressor" (Vlemingckx et al., 1991), but also a "growth suppressor", and its ability to suppress growth depends on its ability to prevent growth factor induced reduction in p27 protein levels.

4.3 Materials and Methods

4.3.1 Cell Lines and Culture Conditions.

The human MCF-7 breast carcinoma cell line and the murine EMT/6 mammary carcinoma cell line were a gift from Dr. Beverly Teicher (Dana-Farber Cancer Institute, Boston, MA). Pc5-T, Pc7-T, Pc10-L and Pc11-L are clones of the parent EMT/6 cell line (previously referred to as E/Pc5-T, E/Pc7-T, E/Pc10-L and E/Pc11-L, respectively) which grow as either "tightly"(T) or "loosely"(L) adherent aggregates in three-dimensional culture and were described previously (St.Croix et al., 1996b). Stable E-cadherin expressing clones of EMT/6 were obtained by lipofectin-mediated (Gibco, NY) co-transfection of 0.5 μg of the plasmid pSV2neo along with 10 μg of the expression vector pBATEM2 containing the mouse E-cadherin gene under the control of a β-actin promoter (M.
Takeichi, Kyoto University, Kyoto, Japan). Following selection in G418-containing medium, G418 resistant clones were screened for E-cadherin expression by immunoblotting. All EMT/6 cells were cultured in Waymouth's MB 752/1 medium, supplemented with 10% FBS. To prevent HA-mediated intercellular adhesion of Pc5-T and Pc7-T cells, 1000 u/ml of bovine testicular hyaluronidase (Worthington Biochemical corporation, Freehold, NJ) was added to three-dimensional cultures at the time of cell plating. EMT/6-E-cadherin transfectants were also grown in the presence of hyaluronidase to ensure the absence of HA-mediated adhesion. Intercellular adhesion of EMT/6-E-cadherin transfectants or E-cadherin positive human cell lines was prevented by treatment with 2 μg/ml of either DECMA-1 antibody (Sigma, Oakville, ON) or SHE78-7 anti-E-cadherin antibody (Zymed, South San Francisco, CA) respectively, at the time of cell plating. For antibodies used in functional studies, sodium azide was removed using dialysis cassettes (Pierce, Rockford, IL). Cells were collected and assayed 48 hours following treatment with hyaluronidase or E-cadherin neutralizing antibodies, unless otherwise stated.

Human L23 large cell lung and BT-20 breast carcinoma cell lines were maintained in RPMI 1640 containing 10% FCS. HT29 and DLD-1 colon and MCF-7 breast carcinoma cell lines were maintained in DMEM medium supplemented with 10% FCS. HBL-100 breast carcinoma cells were grown in McCoy's 5A medium with 10% FCS. The DLD-1 cell line was a gift from T. Sasazuki (Kyushu University, Fukuoka Japan) and the L23 cell line was a gift of P. Twentyman (Lincoln's Inn Fields, London, UK). All other cell lines were purchased from the American Type Culture Collection (Rockville, Maryland). Multicellular aggregates were prepared using the liquid overlay method as previously described (Kobayashi et al., 1993). Briefly, a 4% stock solution of SeaPlaque agarose (FCM Bioproducts, Rockland, Maine) was microwaved, diluted to 1% with heated (56°C)
serum-free medium and coated (0.25 ml) into each well of 24-well plates. 10^5 tumor cells in 1 ml of complete medium were then plated on top of the solidified agarose.

4.3.2 Generation of Cell Lines with Inducible p27

A metallothionein-inducible p27 expression vector, pMT27, was constructed by excising the BglII-EcoRV fragment containing the entire human p27 cDNA from the plasmid pET-3a, (a gift from L. Hengst and S. Reed, Scripps Research Institute, LaJolla, CA) and then ligating it between BamHI and EcoRV of the multiple cloning site of the plasmid pCDNA3-MT. pCDNA3-MT, a gift from J. Filmus (SHSC, Toronto, ON), was generated by replacing the CMV promoter of the plasmid pCDNA3 (Invitrogen, Carlsbad, CA) with the rat metallothionein promoter. Stable p27 inducible clones were obtained by transfecting HT29 or DLD-1 cells with 5μg of pMT27 using standard lipofectin procedures followed by selection in G418-containing medium. G418 resistant clones were screened for inducible p27 by immunoblotting. For induction of the metallothionein promoter, media was supplemented with either 1X ZnCd (100 μM ZnSO₄ and 2 μM CdCl₂) or 2X ZnCd (200 μM ZnSO₄ and 4 μM CdCl₂).

4.3.3 Bromodeoxyuridine (BrdU) Incorporation Assay

EMT/6 cells growing in either three-dimensional culture for twenty four hours or in monolayer culture were pulsed with 5 μM BrdU for a further 24 hours after which cells were immediately rinsed with PBS, trypsinized, rinsed again and fixed with 70% ethanol (4°C) for at least 1 hour. Cells were rinsed with PBS, pelleted, and resuspended in 1 ml of cold 0.1 N HCl/0.7% triton X-100 and left for 10 minutes on ice. After rinsing once with PBS, cells were resuspended in
0.5 ml distilled water and transferred into a 0.5 ml Eppendorf tube already containing 16 μl of 0.1 N HCL. Next, Eppendorf tubes were heated in a PCR machine for 8 minutes at 95°C, and placed immediately on ice. Cells were then transferred to a 5 ml tube, rinsing two times with HNFN buffer (i.e. 10 mM HEPES [pH7.4], 150 mM NaCl, 4% FCS, 0.1% NaN₃) containing 0.5% tween 20, and once with HNFN alone. Pellets were then resuspended in a 1/40 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Cedarlane, Hornby, ON) and left in the dark on ice for 45 minutes. Stained cells were rinsed twice with HNFN tween 20, once with HNFN, pelleted, resuspended in 1 ml of PI solution (50 μg/ml propidium iodide, 10 μg/ml Ribonuclease A in PBS) and left for 1 hour (4°C) in the dark before analysing on an Epics Elite V flow cytometer (Coulter Electronics, Burlington, Ont.).

4.3.4 [³H]-Thymidine Incorporation Assay

Cells growing in monolayer culture were harvested and 10,000 cells/well in a final volume of 100μl of medium was added to 96-well U-bottom Nunc plates (Life Technologies, Burlington, ON). To prevent attachment of cells to the bottom of 96-well plates, the wells had previously been treated with a heated (56°C) solution of 2% poly(2-hydroxyethylmethacrylate) (polyhema) (Aldrich Chemical Company, Inc. Milwaukee WI) in ethanol, which was briefly added to and then removed from the plate, leaving behind a thin film in each well. Intercellular adhesion of E-cadherin positive human cell lines was prevented by supplementing wells with various concentrations of SHE78-7 anti-E-cadherin antibody or isotype matched control IgG antibodies (Sigma). All cells were cultured in their normal growth medium containing 10% FCS, except for the experiment which examined mitogen-dependence of growth stimulation by E-cadherin antibodies. In this experiment, cells were
plated in serum-free medium, or medium containing either 10% FCS or 50ng/ml TGF-α (R&D Systems, Minneapolis, MN). TGF-α signalling was inhibited using 20μg/ml C225 neutralizing anti-EGFR monoclonal antibody (ImClone, New York, NY). After 44 hours of incubation in three-dimensional culture, 2μCi of [³H]-thymidine was added in 50μl of medium to each well. At 48 hours, ie. following a 4 hour pulse, labelled cells were frozen at -20°C. Importantly, [³H]-Thymidine has been shown to penetrate large (400-600μM) spheroids rapidly, reaching full distribution in less than one minute (Nederman et al., 1988). Next, cells were harvested onto filtermats using a Titertek cell harvester 530, and radioactive filtermats were counted using a 1205 beta plate liquid scintillation counter (Fisher Scientific Ltd. Nepean, ON). The rate of DNA synthesis of the groups treated with anti-E-cadherin antibodies was calculated as a fraction of the non-treated controls.

4.3.5 Immunoblotting

Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20mM Tris-HCl [pH7.5], 137mM NaCl, 100mM NaF, 1mM sodium vanadate, 1mM phenylmethyl sulphonyl fluoride and 10μg/ml each of leupeptin and aprotinin). The lysates were sonicated and clarified by centrifugation, resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto Immobilon-P-membranes (Millipore Corporation, Bedford, MA) and blocked in 10% dry milk in TBS-T (Tris Buffered Saline [pH7.6], 0.1% Tween 20). Monoclonal antibodies (mAbs) to p27 and E-cadherin, used for immunoblotting, were obtained from Transduction Laboratories (Lexington, KY), mAb against pRb was from PharMingen (Mississauga, Ontario), polyclonal antibody (pAb) to β-catenin was from Sigma (Oakville, Ontario), pAb to α-catenin was from Zymed Laboratories.
(San Francisco, CA), pAb's to cyclin E, cdk2, cdk4, cdk6, p130, p16, p18, p19, p21 and mAb against mouse cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). DCS-6 mAb, a gift from J Bartek (Danish Cancer Society, Denmark) was used to detect human cyclin D1. After washing in TBS-T the immunoreactive proteins were visualized using either horseradish peroxidase conjugated anti-mouse- or anti-rabbit-IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and the enhanced chemiluminescence (ECL) western blotting detection system (Amersham Life Science, Oakville, Ontario).

4.3.6 Immunoprecipitation

Cells were lysed in NP-40 lysis buffer and immunoprecipitated with antibodies to E-cadherin (Transduction Laboratories) or to cyclin E at 4°C overnight. E-cadherin and cyclin E precipitates were collected for 1 hour on either protein G or protein A Sepharose beads, respectively. After washing four times with lysis buffer, precipitates were resuspended in laemmli SDS sample buffer and resolved by SDS-PAGE. The immunoprecipitated proteins were probed as for the immunoblots except for the use of an alternative secondary antibody against the p27 primary antibody. In this case, an anti-mouse IgG [Fcγ fragment specific] (Jackson ImmunoResearch Laboratories) was used to avoid cross reactivity with the light chain of the immunoprecipitating antibody which ran at a molecular weight similar to p27.

4.3.7 Cyclin-Dependent Kinase Assay

To measure cyclin E-associated cdk2 activity, cells were lysed in ice-cold kinase lysis buffer (150mM NaCl, 50mM Tris [pH 7.6], 0.1% NP40, 1mM PMSF, 10μg/ml leupeptin, 2μg/ml
aprotinin) and 100μg of total protein was immunoprecipitated with anti-cyclin E antibody. After washing the precipitates four times with kinase lysis buffer, and once with reaction buffer (22μM ATP, 7.5mM MgCl₂ and 20mM Tris pH 7.5), 0.25 μg of Histone H1 (Boehringer Mannheim, Laval, Quebec) and 10 μCi of ³²P-γ ATP were added to the reaction buffer for 30 minutes at 37°C. Reaction products were resolved by SDS-PAGE and the gel was dried and exposed to x-ray film.

4.4 Results

4.4.1 E-cadherin mediates intercellular adhesion and growth suppression of EMT/6 cells

To investigate the impact of E-cadherin on contact-dependent growth regulation, we transfected EMT/6 mouse mammary carcinoma cells with a murine E-cadherin expression vector under control of a constitutive β-actin promoter. Neomycin resistant control transfectants, like the EMT/6 parent cell line, were loosely-adherent in three-dimensional culture (see figure 4.1) and lacked any detectable E-cadherin expression by western blot analysis, although α and β-catenin were readily detectable in these cells (see figure 4.2 and data not shown). In contrast, within 24 hours E-cadherin transfectants spontaneously formed compact multicellular aggregates and expressed high levels of E-cadherin protein. To confirm that E-cadherin was responsible for the increased compaction of these clones, suspension cultures were treated with DECMA-1 antibody which blocks mouse E-cadherin mediated adhesion (Vestweber and Kemler, 1985). As shown in figure 4.1, DECMA-1 antibody completely prevented E-cadherin-dependent aggregation, while neutralizing anti-α6 integrin antibody, used as a control, had no effect on adhesion even though these cells express high levels of α6β4 integrin (unpublished observations). Furthermore, treatment with
Figure 4.1  Morphology of EMT/6 cells displaying either E-cadherin or HA-dependent intercellular adhesion in three-dimensional culture.  (A-C) EMT-6 cells transfected with E-cadherin (Ecad), (D) with neomycin vector alone (neo 8) or (E,F) Pc5-T cells with HA-dependent adhesion were cultured with 1000u/ml hyaluronidase (A-D,F), 10μg/ml DECMA-1 antibody (C,E) or 10μg/ml of GoH3 anti-α6-integrin antibody (B) (Bio/can scientific, Mississauga, ON).  Cells were photographed after 24 hours in three-dimensional culture.  (Bar = 50 μm)
Figure 4.2 Immunoblot analysis of E-cadherin and β-catenin in EMT-6 cells. EMT-6 neomycin resistant control transfectants (neo1, neo4), E-cadherin transfectants (Ecad3, Ecad7 or Ecad9) or EMT-6 loosely adherent (Pc10-L and Pc11-L) or tightly adherent (Pc5-T, PcT-7) variants were grown for 48 hours in three-dimensional culture. E-cadherin-dependent adhesion was prevented by treatment with DECMA-1 antibody (Dec) and HA-dependent adhesion was prevented with hyaluronidase (Hy). In the top panel, anti-E-cadherin and anti-β-catenin antibodies detected proteins of 120kd and 95 kD respectively, by western blot analysis. When cell lysates were immunoprecipitated with E-cadherin antibodies and then immunoblotted with anti-E-cadherin and anti-β-catenin antibodies (bottom panel), again bands of 120kd and 95kd were detected.
DECMA-1 did not block HA-dependent aggregation of EMT/6 cells. Conversely, hyaluronidase abolished HA-dependent adhesion but failed to block E-cadherin-mediated adhesion. E-cadherin transfectants were also dispersed by the addition of EDTA, however, such treatment did not affect cells with HA-dependent adhesion (unpublished observations). Thus, using EMT/6 cells, we have generated a convenient model to study the effects of two distinct adhesion systems, one dependent on E-cadherin and the other on HA.

Our previous studies demonstrated that HA-dependent aggregation of EMT/6 variants results in reduced proliferation in three-dimensional culture (St.Croix et al., 1996a). However, this HA-dependent adhesion mechanism is undefined, and may be limited to certain cells types such as EMT-6. To determine if E-cadherin, an important mediator of cell-cell adhesion, could also suppress growth, we pulsed EMT/6-E-cadherin transfectants for 24 hours with bromodeoxyuridine (BrdU), stained cells with FITC-tagged anti-BrdU antibody, and then analysed BrdU-incorporation into DNA by flow cytometry. As shown in figure 4.3A and Table 4.1, only 49-70% of cells from E-cadherin expressing clones incorporated BrdU following a 24 hour pulse in three-dimensional culture. In contrast, under the same conditions, over 95% of loosely-adherent neomycin control transfectants incorporated BrdU. E-cadherin expressing cells that failed to incorporate BrdU were found to be primarily in the G1 phase of the cell cycle (~80-90%) (see figure 3a and data not shown). The remaining non-labelled cells were found in the S- and G2/M-phase, consistent with other reports on spheroids (Allison et. al., 1983; Freyer and Sutherland, 1980). It is unlikely that an insufficient BrdU pulse is responsible for non-labelled S-and G2/M-phase cells, because the BrdU labelling time chosen (24 hours) exceeded the cell division time (~16 hours) (unpublished observations). An independent [$^3$H]-thymidine incorporation assay demonstrated that treatment of EMT/6-E-cadherin
Figure 4.3 A) E-cadherin suppresses growth of EMT-6 cells as measured by BrdU incorporation into DNA. EMT/6 cells transfected with E-cadherin (Ecad9) or neomycin vector alone (neo1) were grown for 48 hours in three-dimensional culture. Twenty-four after plating in three-dimensional culture cells were either untreated (-BrdU) or pulsed with BrdU (+BrdU) for a further 24 hours. After collecting cells were prepared as described in the Materials and Methods, and cell cycle profiles were analysed by flow cytometry. Tightly adherent or hyaluronidase (HYase) dispersed Pc5-T cells were also included as an additional control.
Table 4.1 Ability of cells to aggregate in three-dimensional culture correlates with fraction of proliferating EMT-6 cells according to percentage of BrdU-labeled cells following a 24-hour pulse

<table>
<thead>
<tr>
<th></th>
<th>neo1</th>
<th>neo4</th>
<th>neo7</th>
<th>neo8</th>
<th>Ecad3</th>
<th>Ecad7</th>
<th>Ecad9</th>
<th>Ecad18</th>
<th>Pc10-L</th>
<th>Pc5-T</th>
<th>Pc5-T +HYase$^1$</th>
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<tr>
<td>Aggr.$^*$</td>
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<td>-</td>
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<tr>
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<td>99.7</td>
<td>99.2</td>
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<tr>
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<td>70.3</td>
<td>97.1</td>
<td>53.5</td>
<td>95.8</td>
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</tbody>
</table>

*Ability of cells to form aggregates in three-dimensional culture. (-) indicates loosely or non-adherent-aggregates and (+) indicates tightly adherent aggregates as shown in figure 1.

$^+$Two-dimensional monolayer culture

$^*$Three-dimensional culture

$^1$Hyaluronidase treated
transfectants with 2μg/ml DECMA-1 antibody restored [3H]-thymidine uptake to the level observed in neomycin resistant control transfectants (see figure 4.3B). Importantly, the observed growth inhibition mediated by E-cadherin in three-dimensional cultures was not detected in subconfluent monolayer cultures (see Table 4.1). Likewise 2μg/ml DECMA-1 antibody had no effect on growth of subconfluent monolayers and a very minor effect, if any, on confluent cultures (data not shown).

4.4.2 Growth suppression by E-cadherin is associated with pRb hypophosphorylation, reduced cyclin D1 and elevated p27 levels in EMT/6 cells

To elucidate the mechanism responsible for E-cadherin-dependent growth suppression, an analysis of cell cycle regulators was undertaken. As a control, we included an analysis of EMT/6 variants which display either a loosely-adherent or HA-dependent tightly-adherent morphology in three-dimensional culture. Since the majority of non-cycling tightly-adherent cells were found to be in the G1-phase of the cell cycle (see figure 4.3A), western blots were probed for cell cycle regulators of G1 to S-phase progression. The pRb protein was almost exclusively hyperphosphorylated in all EMT-6 loosely-adherent cell types (see figure 4.4A). This included neomycin resistant control transfectants (neo1 and neo4), DECMA-1 treated E-cadherin transfectants (Ecad3, Ecad7 and Ecad9), loosely-adherent clones (Pc10-L and Pc11-L), and two tightly-adherent clones (Pc5-T and Pc7-T) which had been dispersed with hyaluronidase. In contrast, the hypophosphorylated faster migrating form of pRb was prominent only in tightly-adherent variants displaying either E-cadherin or HA-dependent adhesion. Hypophosphorylation of the pocket protein p130 was also noted in tightly adherent clones (data not shown). For both pRb and p130, changes in phosphorylation status were only evident in cells grown in three-dimensional culture.
Figure 4.3 B) Anti-E-cadherin antibodies reverse E-cadherin-dependent growth suppression of EMT-6 cells. [3H]-thymidine incorporation into DNA was used to measure proliferation of EMT/6-E-cadherin transfectants (Ecad3, Ecad7, Ecad9, and Ecad18) or neomycin resistant control transfectants (neo4, neo7, neo8) in three-dimensional culture. Intercellular adhesion of E-cadherin transfectants was prevented by treatment with 2μg/ml DECMA-1 antibody at the time of plating.
Figure 4.4 A) Changes in cell cycle molecules in response to intercellular adhesion of EMT-6 cells as detected by immunoblotting. Three-dimensional cultures of EMT-6 neomycin resistant control transfectants (neo1, neo4), E-cadherin transfectants (Ecad3, Ecad7 and Ecad9), EMT-6 loosely adherent variants (Pc10-L and Pc11-L) or HA-dependent tightly adherent variants (Pc5-T, PcT-7) were examined. Immunoblots were probed for the cell cycle molecules indicated. E-cadherin-dependent or HA-dependent intercellular adhesion was prevented by treatment with either DECMA-1 antibody (Dec) or hyaluronidase (Hy), respectively. Note changes in pRb phosphorylation and levels of cyclin D1 and p27 in cells allowed to aggregate in three-dimensional culture.
Next, we analysed the expression of cell cycle molecules of potential importance in mediating E-cadherin-dependent growth suppression and hypophosphorylation of pRb and p130. After 48 hours in three-dimensional culture, levels of cdk2, cdk4 and cdk6 were unchanged in all EMT/6 cells, regardless of their capacity to aggregate. Likewise, cyclin E and A protein levels were also unaffected by the degree of intercellular adhesion (figure 4.4A and data not shown). In sharp contrast, levels of cyclin D1 were consistently elevated in EMT-6 cells displaying a loosely-adherent morphology in three-dimensional culture. Interestingly, in monolayer culture, levels of cyclin D1 were unaffected by treatment with DECMA1 or hyaluronidase for 48 hours (data not shown).

We also analysed levels of known CKIs. Although p15 was not detected in EMT-6 cells, p16, p18, p19 and p21 were expressed, but levels of these inhibitors did not correlate with adhesion (see figure 4.4A and data not shown). We were particularly interested in the effect of E-cadherin on p27 protein levels, since previous studies have implicated this CKI in contact-dependent growth inhibition (Hengst et al., 1994; Polyak et al., 1994; Slingerland et al., 1994; St.Croix et al., 1996a). Although the base level of p27 varied somewhat between individual clones, cell lines with a tightly-adherent morphology generally displayed higher p27 levels than did loosely-adherent variants (see figure 4.4A). Furthermore, high p27 levels in tightly-adherent clones displaying either E-cadherin or HA-dependent adhesion were consistently reduced by treatment with either DECMA-1 or hyaluronidase respectively. In contrast, we were unable to detect similar effects in monolayer cultures under similar conditions (data not shown). Thus, increased p27 levels paralleled aggregate compaction and an inverse pattern was observed for cyclin D1, but only in three-dimensional culture.

To determine the kinetics of changes in p27 and cyclin D1 in response to aggregation, levels of these proteins were assayed at various time points after plating cells into three-dimensional
culture. As shown in figure 4.4B, within 6 hours of transferring cells to three-dimensional culture p27 levels were increased in E-cadherin expressing cells. The decrease in cyclin D1, however, occurred between 24 and 48 hours, and its levels fell progressively thereafter. Because activation of p27 by cell-cell contact has been shown to inhibit cyclin E/cdk2 (Hengst et al., 1994; Polyak et al., 1994; Slingerland et al., 1994), we analysed cyclin E associated-histone H1 kinase activity. Cyclin E/cdk2 activity was reduced in E-cadherin transfectants compared to neomycin resistant controls (see figure 4.5A). Likewise, tightly adherent Pc5-T cells displayed low cyclin E-associated kinase activity in three-dimensional culture. Kinase activity was increased when hyaluronidase was added to the medium or when cells were grown in monolayer culture. Immunoprecipitation blotting revealed higher levels of p27 bound to cyclin E in tightly-adherent spheroids compared to their loosely adherent counterparts (see figure 4.5B), while cyclin E levels were unchanged. Taken together, these studies suggest that inhibition of cyclin E/cdk2 by p27 may mediate E-cadherin-dependent growth suppression.

4.4.3 E-cadherin neutralizing antibodies fail to release β-catenin from E-cadherin

The mechanism by which E-cadherin regulates p27 is unclear. Because β-catenin has been implicated in signal transduction (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996), we investigated whether DECMA-1 induces growth of E-cadherin transfectants by stimulating the release of β-catenin bound to E-cadherin at the cell surface. As shown in figure 4.2, levels of endogenous β-catenin protein were elevated in all EMT/6-E-cadherin transfectants, an observation consistent with other reports (Papkoff, 1997) and most likely due to stabilization of β-catenin bound
<table>
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**Figure 4.4 B)** Time course demonstrating changes in p27 and cyclin D1 protein levels as cells aggregate in three-dimensional culture. Cells transfected with E-cadherin (Ecad3) grown in the presence or absence or 2μg/ml DECMA-1 antibody for the indicated times were probed for p27 and cyclin D1 by immunoblotting. At time=0 cells were transferred from two- to three-dimensional culture.
Figure 4.5 A) Reduced Cyclin E-associated kinase activity in aggregated cells. Cyclin E-associated cdk2 kinase activity, as measured by phosphorylation of Histone H1 substrate, in neomycin resistant control transfectants (neo1, neo4, neo8) or E-cadherin transfectants (Ecad1, Ecad9, Ecad18) grown in three-dimensional culture. Also included were Pc5-T cells grown in monolayer (2-D) culture, or in three-dimensional culture in the presence or absence of hyaluronidase (Hy).
Figure 4.5 B) Increased levels of p27 bound to cyclin E in tightly adherent EMT-6 spheroids. The cell lines and conditions used were the same as in figure 4 A). After immunoprecipitating cyclin E from tightly or loosely adherent aggregates, levels of cyclin E bound p27 were detected by immunoblotting. Cell-free lysis buffer was used for the control sample. To avoid cross-reactivity with the light chain from the immunoprecipitating antibody which also ran at ~27 Kd, a secondary anti-mouse IgG antibody was used which only recognized the Fcγ fragment.
to E-cadherin at the cell surface. β-catenin levels in E-cadherin immunocomplexes were unaffected by DECMA-1 antibody treatment (see figure 4.2). Likewise, levels of E-cadherin bound to immunoprecipitated β-catenin were unchanged by antibody treatment (data not shown). These results suggest that changes in the association of β-catenin with E-cadherin are not involved in growth stimulation mediated by E-cadherin neutralizing antibodies.

4.4.4 *Endogenous E-cadherin mediates intercellular adhesion, growth suppression and upregulation of p27 in human carcinoma cell lines*

To ascertain the role of E-cadherin in contact-dependent growth inhibition of E-cadherin expressing human cell lines, we analysed the effect of SHE78-7, an anti-human E-cadherin neutralizing antibody (Watabe et al., 1994), on various human colon (HT29 and DLD-1), breast (MCF-7 and BT-20) and lung (L23) carcinoma lines. Similar to our observations with EMT6-E-cadherin transfectants, SHE78-7 antibody was highly effective at preventing E-cadherin-mediated adhesion (see figure 4.6 and data not shown). In contrast, intercellular adhesion of these human cells was unaffected by isotype-matched control IgG, and SHE78-7 failed to block adhesion of tightly-adherent HBL-100 breast carcinoma cells which lack any detectable E-cadherin expression (see figure 4.6 and 4.8).

We next asked whether blocking E-cadherin function could stimulate the growth of these human cell lines. As shown in figure 4.7, addition of SHE78-7 to the culture medium stimulated the uptake of [³H]-thymidine by each of the colon, breast and lung cancer cell lines tested in a dose-dependent manner. The effect of SHE78-7 on growth paralleled its effect on intercellular adhesion. Interestingly, antibody stimulation reached a plateau at concentrations of 1-2 μg/ml, presumably due
Figure 4.6  Morphology of human E-cadherin positive cell lines in three-dimensional culture. E-cadherin positive (A,B) HT29 colon, (C,D) L23 lung, (E,F) BT20 breast, or E-cadherin negative (G,H) HBL100 breast carcinoma cell lines were cultured in the presence of 2μg/ml SHE78-7 anti-E-cadherin antibodies (B,D,F,H) or isotype-matched control IgG antibodies (A,C,E,G). Cells were photographed 24 hours after plating. Bar = 50 μM.
Figure 4.7 E-cadherin neutralizing antibodies stimulate the growth of human E-cadherin positive cell lines in three-dimensional culture. Colon (HT29, DLD-1), lung (L23) and breast (BT20, MCF-7, HBL100) carcinoma cell lines were cultured for 48 hours in the presence of SHE78-7 anti-E-cadherin antibodies (—○—) or isotype-matched control IgG antibodies (—▲—). After pulsing with $[^3]$H]-thymidine, radioactivity incorporated into DNA was measured and expressed as a percentage of non-treated control cells. Note that E-cadherin antibodies only stimulate the growth of cell lines which display E-cadherin-mediated intercellular adhesion.
to saturation of antibody binding sites at the cell surface. The antibody was completely non-toxic even at concentrations of 50 μg/ml (data not shown). Again, E-cadherin negative HBL-100 cells were unaffected by SHE78-7.

To determine whether the effects of E-cadherin on pRb, p130, cyclin D1 and p27 in murine EMT-6 cells were similar in human cells, we analysed levels of these proteins in colon (HT29 and DLD-1), lung (L23) and breast (BT20 and HBL100) carcinoma cell lines. Cells grown for 48 hours in three-dimensional culture in either the presence or absence of SHE78-7 antibody were analysed by western blot analysis. Similar to our previous results with EMT/6-E-cadherin transfectants, when E-cadherin mediated adhesion of human cells was abrogated by SHE78-7, there was a shift to increased phosphorylation of pRb and p130 (see figure 4.8). However, unlike our initial experiments, addition of E-cadherin neutralizing antibodies did not result in a detectable increase in cyclin D1 by 48 hours. In each of the E-cadherin positive cell lines allowed to aggregate, p27 levels increased from ~2.5-fold to ~15-fold, depending on the cell line. Importantly, E-cadherin negative HBL100 cells were unaffected by E-cadherin antibody treatment.

4.4.5 Enforced upregulation of p27 substitutes for E-cadherin-dependent growth suppression

To assess further the role of p27 in E-cadherin-mediated growth suppression we transfected HT29 cells with a metallothionein-inducible human p27 expression vector. One of the transfectants, H/MT27, which was found to produce high levels of p27 in response to treatment with zinc and cadmium, was plated into three-dimensional culture in the presence or absence of SHE78-7 E-cadherin neutralizing antibody. Importantly, by titrating the concentration of zinc and cadmium we were able to accurately control the level of p27 in cells. Thus, in HT29 cells in which intercellular
Figure 4.8 Changes in cell cycle regulators in response to E-cadherin mediated adhesion of human carcinoma cell lines. Immunoblots of colon (HT29, DLD-1), lung (L23) and breast (BT20) carcinoma cell lines were probed for p27, pRb, p130 and cyclin D1. E-cadherin-dependent intercellular adhesion was prevented by treatment with 2μg/ml of SHE78-7 antibody (+SHE).
adhesion was prevented by SHE78-7 antibody treatment, we could enforce expression of the same amount of p27 that was present in tightly adherent cells grown in the absence of SHE78-7. As shown in figure 4.9, enforced elevation of p27 in SHE78-7 treated cells restored approximately 50% of the reduction of [3H]-Thymidine uptake observed in tightly adherent control cells. In contrast, neither the HT29 parent population or neomycin resistant control transfectants were significantly affected by treatment with zinc and cadmium. We have observed similar results for pMT27 transfectants of DLD-1 cells (data not shown). These results suggest that p27 plays a major role in growth suppression mediated by E-cadherin, but other factors are also likely to be involved.

4.4.6 E-cadherin suppresses growth by inhibiting down regulation of p27 by growth factors

Recent studies have provided circumstantial evidence that E-cadherin-mediated adhesion may suppress growth by inactivating receptor tyrosine kinase (RTK) activity. Before testing this directly, we asked whether or not E-cadherin neutralizing antibodies could stimulate growth in the absence of mitogens in the culture medium. HT29 was used since this cell line is E-cadherin positive and continues to grow, albeit more slowly, in the absence of fetal calf serum (FCS) (see figure 4.10). When HT29 cells were treated with SHE78-7 in serum-free medium, p27 levels were unchanged and [3H]-thymidine uptake was unaffected even though intercellular adhesion was prevented. When 10% FCS was added to the medium alone, only a 2-fold increase in [3H]-thymidine uptake of compact aggregates was observed. Addition of 50ng/ml TGF-α was moderately more potent, causing approximately 3-fold stimulation of [3H]-thymidine uptake. These were optimal growth stimulatory concentrations of FCS and TGF-α in three-dimensional culture as determined by dose-response studies (data not shown). However, if E-cadherin mediated adhesion
Figure 4.9 Enforced expression of p27 inhibits cell growth in the presence of E-cadherin neutralizing antibodies. HT29 cells transfected with a metallothionein-inducible p27 expression plasmid (H/MT27), neomycin vector alone (H.neo), or non-transfected HT29 cells (HT29), were grown in three-dimensional culture for 48 hours in the absence (Control) or presence of SHE78-7 antibody (+SHE). For induction of the metallothionein promoter, cultures were supplemented with either a one (1X) or two times (2X) concentration of Zinc and Cadmium (ZnCd). \[^3\text{H}\]-thymidine incorporated into DNA was measured and expressed as fold increase in counts over non-treated control cells. The corresponding levels of p27 as determined by immunoblotting are shown in the inset.
Figure 4.10 Mitogens are required for growth stimulation in the presence of E-cadherin neutralizing antibodies. Non-treated HT29 cells (control) were grown in serum-free medium for 48 hours. Treated cells were grown in the presence of 2μg/ml SHE78-7 antibody (+SHE), 10% FCS (+FCS) or 50ng/ml TGF-α (+TGF-α), either alone or in various combinations. To prevent TGF-α activity, cultures were treated with c225 neutralizing anti-EGRF antibody. After pulsing with [³H]-thymidine, radioactivity incorporated into DNA was measured and expressed as fold increase in counts over non-treated control cells. The corresponding levels of p27 (inset) were determined by immunoblotting.
was blocked by SHE78-7, growth stimulation by FCS was even greater, about 5-fold, while TGF-α stimulated [3H]-thymidine uptake almost 10-fold compared to serum-free controls. These effects on [3H]-thymidine uptake were clearly synergistic because SHE78-7 antibody had no effect on proliferation by itself in the absence of mitogens. When TGF-α, FCS and SHE78-7 were all added simultaneously, an even larger (~14-fold) growth stimulation was noted (data not shown). Importantly, the growth stimulation observed following treatment with E-cadherin antibodies was associated with a reduction in p27 protein. Furthermore, the ability of TGF-α to both stimulate growth and reduce p27 protein levels could be completely abrogated by adding C225 epidermal growth factor receptor (EGFR) blocking antibody to the culture medium. In contrast, treatment with C225 had no effect on FCS-induced growth stimulation. We conclude that E-cadherin inhibits growth of HT29 cells by inhibiting TGF-α effects on EGFR kinase activity or other unknown mitogenic pathways. Thus, E-cadherin mediated adhesion does not affect p27 levels directly, but indirectly, and this can occur through inhibition of RTK activity.

4.5 Discussion

It has long been recognized that non-transformed cell lines in culture are contact inhibited when they reach confluence in monolayer tissue culture. However, it has been shown only recently using normal fibroblasts and epithelial cells, that intercellular adhesion molecules such as NCAM and E-cadherin respectively, are involved in this process (Aoki et al., 1991; Kandikonda et al., 1996; Takahashi and Suzuki, 1996). Although loss of contact inhibition is one of the classical hallmarks of transformation, many tumor cell lines also appear to maintain a degree of contact inhibition, which we refer to as contact-dependent growth inhibition/suppression. This phenomenon, which
appears to be most pronounced when cells are cultured as three-dimensional masses, may reflect an incomplete loss of contact inhibition during the transformation process.

4.5.1 *E-cadherin mediates contact-dependent growth inhibition of tumor cells*

The present study suggests that, as for normal cells, E-cadherin is also involved in contact-dependent growth inhibition of carcinoma cell lines which maintain functional E-cadherin. This was shown using E-cadherin neutralizing antibodies which simultaneously disrupted adhesion and stimulated the growth of several E-cadherin positive breast, colon and lung carcinoma cell lines grown in three-dimensional culture. Similar effects of E-cadherin on growth were obtained by Watabe and co-workers, in this case using an α-catenin expressing variant of the F9 small cell lung carcinoma cell line which has an intact E-cadherin adhesion system (Watabe et al., 1994). Interestingly, this cell line displays very weak attachment to tissue culture flasks, growing mostly as free floating multicellular spheroids. Furthermore, transfection of E-cadherin into non-adherent, E-cadherin-negative carcinoma cell lines has been shown to cause morphological reversion to an adherent epithelial phenotype and to decrease tumor growth rate when the cells are injected subcutaneously into mice (Miyaki et al., 1995; Navarro et al., 1991). Herminston and Gordon have shown that inactivation of endogenous E-cadherin in intestinal crypt cells using a truncated N-cadherin (which presumably acts as a dominant negative) leads to the formation of adenomas (Hermiston and Gordon, 1995b). These studies, in addition to our own, provide strong evidence that E-cadherin is not only a suppressor of invasion (Vleminckx et al., 1991), but also has the ability to suppress growth. Importantly, this effect of E-cadherin appears to be most pronounced in vivo or in confluent monolayer or three-dimensional cell cultures.
4.5.2 Increased levels of p27 are involved in E-cadherin mediated growth suppression

To elucidate the mechanism(s) responsible for E-cadherin-dependent growth suppression, we have investigated the effect of E-cadherin-mediated adhesion on cell cycle regulators. Our analysis revealed that the CDK inhibitor, p27\(^{kip}\), was elevated in a panel of cell lines which manifest E-cadherin-dependent adhesion, compared to the same cell lines rendered loosely adherent by treatment with anti-E-cadherin antibodies. Further studies demonstrated that this increase in p27 is responsible, at least in part, for E-cadherin-dependent growth inhibition. First, E-cadherin was shown to suppress cyclin E-associated kinase activity, through an increased binding of p27 to the cyclinE/cdk2 complex. Second, when cells disrupted by E-cadherin neutralizing antibodies were engineered to express p27 levels corresponding to those observed in adherent spheroids, antibody stimulated proliferation was repressed by approximately 50%. Consistent with our results, when p27 activity was first identified, it was found to be present in contact-inhibited monolayer cultures of MvLu epithelial cells (Polyak et al., 1994; Slingerland et al., 1994). Interestingly, MvLu cells express E-cadherin at sites of cell-cell contact (Brady-Kalnay et al., 1993). This study, together with our previous one (St.Croix et al., 1996a), demonstrates the involvement of p27 in both E-cadherin and HA-dependent signalling pathways. Thus, distinct adhesion mechanisms may impinge on the same signal transduction pathways regulating the cell cycle machinery.

While the present study clearly demonstrates the importance of p27 in growth suppression mediated by E-cadherin, a portion of the growth inhibition, roughly half, could not be accounted for by p27 expression levels. Although our replacement strategy may have resulted in some quantitative inaccuracies, other factors are likely to be involved in E-cadherin-dependent growth inhibition. When E-cadherin positive cell lines were allowed to aggregate, we also observed
hypophosphorylation of pRb and p130, and in tightly adherent EMT/6 cells, cyclin D1 was reduced. The loss of pRb and p130 phosphorylation in adherent cells is most likely a consequence of reduced cyclin/cdk activity as cells arrest in G1/G0. However, E-cadherin-dependent downregulation of cyclin D1 may act synergistically with p27 upregulation to mediate G1 arrest. Effects of adhesion on cyclin D1 may be cell type dependent because, in our panel of human E-cadherin positive cell lines, cyclin D1 levels appeared unaffected by treatment with E-cadherin neutralizing antibodies. It is possible, however, that other D-type cyclins are involved or that cyclin D1 turnover is more rapid in antibody treated cells, perhaps through ubiquitin-mediated proteolysis (Diehl et al., 1997). While further studies are necessary in order to address these possibilities, a connection between cyclin D and E-cadherin is intriguing given the relationship between growth factor dependence of E-cadherin-mediated growth suppression, and the known ability of growth factors to regulate cyclin D turnover (Sherr, 1995).

4.5.3 Mitogenic pathways are required for E-cadherin dependent growth suppression and elevation of p27 protein levels

During the course of these studies, we became interested in understanding signal transduction pathway(s) by which E-cadherin regulates p27. Initially, we hypothesized that E-cadherin may sequester β-catenin at the cell surface, thereby preventing its ability to translocate to the nucleus and alter the transcription of growth promoting genes. However, in preliminary experiments we could find no evidence to support this hypothesis, and our later results suggested that classical mitogenic pathways are necessary for growth stimulation in the presence of E-cadherin neutralizing antibodies. Indeed, under serum free conditions, E-cadherin neutralizing antibodies were unable to stimulate
growth or reduce p27 levels in HT29 cells even though intercellular adhesion was completely prevented. However, E-cadherin neutralizing antibodies synergized with FCS or TGF-α, increasing their capacity to stimulate growth of HT29 cells by 2.5- and 3.2-fold respectively. Importantly, the effect of TGF-α on both p27 levels and growth could be completely abolished by adding C225 neutralizing antibody against the EGFR to cell cultures. Thus, E-cadherin dependent adhesion regulates p27 levels and proliferation indirectly by preventing the mitogenic activity of soluble growth factors.

It is unclear exactly how adhesion mediated by E-cadherin prevents signalling through RTKs such as the EGFR, which in turn leads to increased levels of p27 protein. Several groups have shown that ligand-induced phosphorylation of RTKs such as the EGFR or Erb-B2 is lost upon cell-cell contact even in the presence of excess ligand (Lichtner and Schirrmacher, 1990; Mansbridge et al., 1992). In one study, E-cadherin antibodies were shown to prevent contact-inhibition and restore EGFR phosphorylation in response to EGF (Takahashi and Suzuki, 1996). Because phosphorylation of RTKs can be rescued upon cell-cell contact by addition of phosphatase inhibitors (Mansbridge et al., 1992; Sorby and Ostman, 1996), contact-dependent RTK inactivation is thought to be a consequence of endogenous phosphatase activity. Indeed, several receptor protein tyrosine phosphatases (RPTPs) that span the extracellular membrane have been shown to be upregulated and activated by cell-cell contact (Gaits et al., 1995; Gebbink et al., 1995; Ostman et al., 1994; Pallen and Tong, 1991) and in some cases co-localize with cadherin-catenin complexes (Balsamo et al., 1996; Kypta et al., 1996; Wang et al., 1996). Tight adhesion mediated by E-cadherin or other adhesion mechanisms may serve to strengthen interactions between adjacent RPTPs ensuring high continuous phosphatase activity. This in turn may lead to dephosphorylation of the EGFR and a build up of p27
Interestingly, β-catenin has been found by co-immunoprecipitation studies to link cadherin-catenin complexes to several receptor tyrosine kinases such as the EGFR, c-erbB2, and possibly c-met. (Hoschuetzky et al., 1994; Kanai et al., 1995; Shibata et al., 1996; Shibata et al., 1996). Whether or not adhesion mediated by E-cadherin can inhibit RTK activity through such an interaction is unclear. However, in at least some systems the opposite appears to be true, that is, ligand induced activation of RTKs appears to destabilize adhesion (Shibamoto et al., 1994; Shiozaki et al., 1995). It has been proposed that this effect may be due to the phosphorylation of β-catenin by RTKs since phosphorylated β-catenin is not found in cadherin-catenin complexes bound to the actin cytoskeleton (Hoschuetzky et al., 1994). Although plausible, evidence for this hypothesis is mostly circumstantial, and one study demonstrated that phosphorylation of β-catenin is not required to shift cadherin-based adhesion from the strong to weak state (Takeda et al., 1995). Furthermore, we and others have found that adhesion is unaffected by the presence of mitogens such as TGF-α and EGF, arguing against a role for RTK induced destabilization of adhesion (Shibamoto et al., 1994). Instead, it has been proposed using sparse monolayer cultures of normal epithelial cells, that RTK phosphorylation of β-catenin may inhibit binding of β-catenin to α-catenin thereby preventing an inappropriate connection to the underlying cytoskeleton before cell-cell contact occurs (Takahashi and Suzuki, 1996). Once cell-cell contact occurs, dephosphorylation of RTKs and/or β-catenin by phosphatases may effectively block phosphorylation of β-catenin.

The mechanisms by which mitogen activated RTK signaling pathways regulate p27 is an area of active investigation. Several studies have demonstrated that p27 levels are reduced in epithelial
cells, endothelial cells, fibroblasts and T-cells in response to growth factors such as EGF, VEGF, PDGF and IL2 (Agrawal et al., 1996; Coats et al., 1996; Peng et al., 1996; Shankland et al., 1997; Watanabe et al., 1997; Winston et al., 1996). Furthermore, depletion of p27 using antisense strategies partially prevents withdrawal from the cell cycle in response to mitogen deprivation (Coats et al., 1996; Rivard et al., 1996). Exit from quiescence after addition of growth factors has been shown to decrease p27 protein levels due to increased ubiquitin-mediated degradation of p27 (Pagano et al., 1995), but other translational mechanisms such as a decreased rate of p27 synthesis (Agrawal et al., 1996; Hengst and Reed, 1996) or a decrease in transcription (Peng et al., 1996) have also been described. In fibroblasts, ras signaling through the MAP kinase pathway has been implicated in enhanced p27 degradation (Aktas et al., 1997; Kawada et al., 1997; Takuwa and Takuwa, 1997). Interestingly, p27 is phosphorylated by MAP kinase in vitro and phosphorylated p27 cannot bind to and inhibit cdk2 (Alessandrini et al., 1997; Kawada et al., 1997). Phosphorylation of p27 by cyclin E-cdk2 may also regulate p27 destruction in response to mitogens (Sheaff et al., 1997; Vlach et al., 1997).

Based on our studies of HT29 cells, it appears that signals from at least three independent sources impinge on the cell cycle machinery to regulate proliferation of tumor cells. First, unlike non-transformed cells, these cells are anchorage-independent and grow autonomously in the absence of exogenously added growth factors. Signalling in this case appears to be constitutive and may be the result of genetic alterations in oncogenes or tumor suppressor genes. Indeed, HT29 cells are known to have mutated p53 and APC genes (Huang et al., 1994). Second, addition of mitogens, such as FCS or TGF-α to the growth medium enhances the rate of proliferation of HT29 tumor cells. Finally, results of this study demonstrate that adhesion mediated by E-cadherin can also regulate
responsiveness of cells to growth factors such as TGF-α. In three-dimensional culture, E-cadherin mediated adhesion was the most powerful regulator of growth, repressing mitogenic growth stimulation more than 3-fold.

4.5.4 Suppression of tumor growth by E-cadherin is incomplete

An important finding from these studies is that contact-dependent growth suppression of tightly adherent tumor cells, in contrast to normal cells, is incomplete. In fact, we found it difficult to detect any effect of E-cadherin neutralizing antibodies on high cell density monolayer cultures of E-cadherin positive cells, unlike other studies which have employed normal epithelial cells (Kandikonda et al., 1996; Takahashi and Suzuki, 1996). This discrepancy may be explained by a reduced sensitivity of tumor cells to undergoing contact-dependent growth inhibition. However, in a tumor-like three-dimensional context, all E-cadherin positive tumor cell lines displayed a significant, albeit still incomplete, reduction in cell growth. The enhanced ability to detect contact-dependent growth inhibition in three-dimensional culture may be due to the fact that under such conditions tumor cells are exposed to a relatively greater area of cell surface contact, and consequently may receive a stronger growth inhibitory signal. Whatever the reasons, these results suggest a need for caution when studying tumor cell growth exclusively in monolayer culture.

Given the ability of E-cadherin to suppress both growth and invasion, carcinomas which lose E-cadherin-mediated adhesion either through direct loss of E-cadherin, or indirectly through loss of α- or β-catenin, may acquire a more aggressive behaviour. Partial loss of contact-dependent growth inhibition of tumor cells which maintain E-cadherin function may also help to explain why such E-cadherin positive carcinomas are still capable of growing in vivo (Miyaki et al., 1995; Navarro et
In that regard, it will be of interest to understand what causes partial loss of contact inhibition in cell lines where E-cadherin-dependent adhesion is observed. One possibility is that transformation induces changes in the levels or activities of components of the E-cadherin system itself resulting in weaker E-cadherin mediated adhesion, for example through phosphorylation of E-cadherin (Stappert and Kemler, 1994) or β-catenin (Shibamoto et al., 1994). Alternatively, incomplete E-cadherin-dependent growth inhibition may result from loss of an alternative adhesion mechanism (such as HA-dependent adhesion), or the acquisition of constitutive intracellular mitogenic signalling.

The fact that partial E-cadherin function is maintained in certain tumors and is not selected against, despite its ability to inhibit growth, implies that some level of E-cadherin function may be advantageous to tumor cells. For example, E-cadherin has been shown to protect cells from undergoing apoptosis or programmed cell death (Hermiston and Gordon, 1995a; Hermiston and Gordon, 1995b). Thus, in addition to growing more slowly (Miyaki et al., 1995; Navarro et al., 1991), solid tumors with functional E-cadherin may also be resistant to apoptosis induced by environmental stress (Helmlinger et al., 1997), or cytotoxic anticancer therapy (Dimanche-Boitrel et al., 1994; St.Croix and Kerbel, 1997). Even when E-cadherin adhesion is completely disabled due to a loss of E-cadherin, α-catenin, or β-catenin, tumor cells may still display partial contact dependent growth inhibition due to the presence of other adhesion mechanisms, such as the HA-dependent mechanism observed in our EMT/6 cells. Indeed, whereas E-cadherin expression is frequently down regulated with tumor progression, many other adhesion molecules are upregulated. These include CD44, CEA (carcinoembryonic antigen) and ICAM-1 (Johnson, 1991; Jothy et al., 1995).

In summary, we demonstrate using tumor cells that the cdk inhibitor p27 plays a significant
role in E-cadherin-dependent growth inhibition. Growth inhibition by cell-cell adhesion may also help to explain why tumor cells, despite harbouring multiple mutant oncogenes and tumor suppressor genes, often contain low growth fractions in vivo. E-cadherin can inhibit growth by inhibiting the activity of mitogenic pathways such as the EGFR which in turn regulate the level of p27 in cells. E-cadherin-dependent upregulation of p27 may also help to explain the widely observed phenomenon of contact-inhibition of normal epithelial cells.
Chapter 5

Discussion and Future Directions
5.1 Discussion

The preceding three chapters of this thesis provide experimental evidence that intercellular adhesion can have a significant impact on both tumor cell proliferation and resistance to anticancer therapy. This was first demonstrated in experiments summarized in chapter 2, where use was made of a panel of EMT-6 tightly-adherent and loosely-adherent clones, as well as the anti-adhesive hyaluronidase. Using this model, it was demonstrated, using standard colony formation assays, that an increase in intercellular adhesion was sufficient to confer resistance to the alkylating agent 4-HC. Hyaluronidase was also found to be effective at sensitising tightly-adherent variants in three-dimensional culture to γ-irradiation (see figure 5.1). Most importantly, using an in vivo ascites tumor model it was shown that hyaluronidase could disperse tumor cell aggregates and sensitize tumor cells to cyclophosphamide in vivo.

5.1.1 The HA-Dependent Adhesion Mechanism

Although the exact molecular mechanism regulating adhesion in tightly-adherent EMT-6 variants remains elusive, the disaggregating effect of hyaluronidase implies the involvement of HA and HA receptors. Initial experiments with these clones suggested there are at least two independent adhesion processes which take place in three-dimensional culture. The initial adhesion event occurs rapidly when cells are placed in three-dimensional culture and is probably shared between the tightly- and loosely-adherent EMT-6 variants. This adhesion appeared weak as cells were easily dispersed from such aggregates by mechanical pipetting. When loosely- or tightly-adherent variants were stained with red or green non-toxic fluorescent dyes respectively, and then plated together in three-dimensional culture, by six hours, the tightly adherent cells were found to recognize and
Figure 5.1  Hyaluronidase sensitizes E/Pc7-T cells to $\gamma$-IR in vitro. E/Pc7-T cells were grown in three-dimensional culture in the presence or absence of hyaluronidase. After two days, cells were irradiated and 24 hours later plated in a colony formation assay. To ensure homogeneous microenvironmental influences, culture conditions were designed so that cellular clumps never exceeded 10-20 cells in number.
homotypically bind to one another (see figure 5.2). This secondary 'homophilic' adhesion mechanism appears to be much stronger than the first since, by 48 hours it is not possible to disperse such clumps mechanically under the same conditions or in the presence of EDTA. We believe that this latter adhesion mechanism, which is hyaluronidase-sensitive and divalent cation-independent, is responsible for the compaction we have observed in the drug-resistant variants and tightly-adherent clones. This adhesion mechanism responsible for HA-dependent aggregation is unlikely to be CD44, since KM201 and KM114, two neutralizing antibodies which block binding of HA to mouse CD44 (Zheng et al., 1995), were unable to prevent adhesion of tightly-adherent variants (unpublished observations). Recently, the homophilic cell surface adhesion molecule ICAM-1 was also shown to possess HA-binding activity (McCourt et al., 1994). However, we were unable to detect ICAM-1 expression in EMT-6 cells (BSC, unpublished observations).

5.1.2 Cell Adhesion and Multicellular Drug Resistance

One of the objectives of our initial studies on multicellular drug resistance was to investigate possible mechanisms by which cell-cell aggregation contributes to the drug resistance phenotype. The fact that loosely-adherent cells grew significantly faster than tightly-adherent variants, in combination with the fact that cyclophosphamide is known to preferentially target rapidly dividing cells suggested that the growth fraction may be an important factor regulating multicellular drug resistance. This possibility was strengthened by experiments which ruled out insufficient drug penetration into the spheroid core as a possible mechanism of resistance. Nevertheless, it is unclear why cyclophosphamide is most active against rapidly dividing cells. Further insight into possible mechanisms regulating the multicellular drug resistance phenotype came from an analysis of the
Figure 5.2 Assessment of homotypic aggregation by fluorescent cell tracking of mixtures of E/CTX and E/Pc10-L cells in three-dimensional culture. Cells were stained green or red with the non-toxic fluorescent stains PKH2 and PKH26, respectively, after which cells were plated into three-dimensional culture. A), B), C), and D) show a 50-50 mixture of E/CTX cells (stained green) and E/Pc10-L cells (stained red) after 0, 6, 24 and 48 hours, respectively under an FT510 (green) filter. E) and F) show the same field as in D) except under an FT580 (red) filter (E) or normal illumination (F). Note the formation of large homotypic spheroidal clusters of E/CTX cells (arrowheads) amongst the loosely adherent E/Pc10-L cells by 48 hours.
effect of adhesion on cell cycle perturbations following anticancer therapy.

5.1.3 Cell Adhesion Suppresses the G2/M Arrest Following Exposure to Anticancer Agents

Most, if not all, anticancer agents are able to induce cell cycle arrest at one or more phases of the cell cycle. It has been hypothesized that cell cycle arrest at a particular phase, often referred to as a 'checkpoint', allows cells an opportunity to monitor (or check) DNA integrity before resuming cell cycle progression. For example, it has been postulated that the G1 cell cycle checkpoint allows cells to monitor and repair any DNA damage before DNA replication begins. If too much DNA damage has occurred (i.e. above a certain threshold level), the cell may respond by undergoing apoptosis. In a similar manner, the G2/M arrest may allow cells an opportunity to monitor DNA integrity before executing mitosis.

A few studies have shown that cells in multicellular spheroids are highly resistant to drug-induced cell cycle perturbations while the same cells grown in monolayer culture typically arrest in G2/M (Hinz and Dertinger, 1983; Sano et al., 1983; St. Croix et al., 1996a; Frankel et al., 1997). A key factor regulating this G2/M arrest following drug exposure appears to be the level of intercellular adhesion. For example, tightly adherent EMT-6 variants resisted cell cycle arrest compared with the same cells dispersed with hyaluronidase (see chapter 2). Reduction in the G2/M arrest can be explained, at least in part, by a decreased number of cycling cells in tightly adherent spheroids. Thus, a high proportion of cells in the G1 phase of the cell cycle found in tightly adherent spheroids may act analogously to a G1 checkpoint and allow cells time to monitor and repair DNA damage before DNA replication occurs. Although a decreased number of cycling cells in tightly adherent spheroids likely accounts for a large proportion of their resistance to undergoing G2/M
arrest, other factors may also be involved. For example, in tightly adherent spheroids the G2/M arrest could be significantly enhanced following drug exposure by treating cells with Nocodazole, a G2/M phase blocker. Thus, cycling cells in tightly adherent spheroids may also be intrinsically resistant to undergoing G2/M arrest.

In monolayer cultures G2/M arrest is widely observed following exposure to many anticancer agents including cyclophosphamide. In contrast, when solid tumors are treated with the same agents, such cell cycle perturbations are frequently not observed or, if they are, they are small (D'Incalci et al., 1983; Ferrari et al., 1989; Jackel and Kopf-Maier, 1991; Wennerberg et al., 1984; Remvikos et al., 1993b; Briffod et al., 1992; Spyatos et al., 1992). An increased G2/M arrest in responsive compared with non-responsive tumors has been noted in breast cancer patients and human tumor xenografts following therapy with cyclophosphamide and other chemotherapeutic agents (D'Incalci et al., 1983; Ferrari et al., 1989; Jackel and Kopf-Maier, 1991; Wennerberg et al., 1984; Remvikos et al., 1993b; Briffod et al., 1992; Spyatos et al., 1992). In some cases a diminished response in vivo may be explained by insufficient exposure of tumor cells to drug due to drug delivery problems or enhanced host-dependent drug detoxification or elimination. In other cases repressed cell cycle perturbations may be due to the development of tumor drug resistance. The studies reported herein suggest that the degree of intercellular adhesion in a solid tumor mass may also affect the degree cell cycle arrest observed in response to therapy.

5.1.4 The Effect of Adhesion, Cell Cycle Kinetics and p27 on Drug Resistance

The high proportion of G1 cells in tightly adherent compared with loosely adherent aggregates appears to be regulated by p27 since depletion of this CKI increases the S-phase fraction
and enhances drug or radiation induced G2/M arrest (see chapter 3). It is unclear, however, whether an adhesion/p27-dependent accumulation of cells in G1 is necessary for resistance to DNA damaging agents. It is possible that G0/G1 cells have an enhanced capacity to repair DNA lesions through a process known as potentially lethal damage repair (PLDR) (Mendonca et al., 1990; Little et al., 1973). Adherent, slowly proliferating spheroid cells may also be less susceptible to DNA damage due to altered DNA "packaging" (Olive and Durand, 1994). Resistance may also be due to cell-cell or cell-ECM interactions which protect cells from apoptosis (Meredith et al., 1993; Frish and Francis, 1994; Bates et al., 1994). If this is the case, then it is not clear whether the effects of adhesion on cell cycle are also necessary for the resistance phenotype. It is noteworthy that antisense oligonucleotides against p27 in addition to stimulating growth and chemosensitizing tumor cells also abolished intercellular adhesion.

From a molecular viewpoint, increased levels of bcl-2 (Aoshiba et al., 1997) or decreased SAPK activity in response to cell-cell contact (B. Zanke, personal communication) may also be necessary for apoptosis resistance, although this has yet to be formally demonstrated. Changes in bcl-2, SAPK or p27 may also help to explain the so called "contact effect" or "confluence dependent resistance", two terms used to describe the relative resistance of spheroids or confluent monolayer cultures to a variety of cytotoxic agents (Sutherland, 1988; Olive and Durand, 1994; Dimanche-Boitrel et al., 1992; Dimanche-Boitrel et al., 1993). Regardless of the mechanisms responsible for resistance, our studies suggest that p27 antagonists [or alternatively antiadhesives (see section 5.7)] may be useful chemosensitizers in combination with current anticancer therapy.
5.1.5 Targeting p27 as a Therapeutic Strategy

Mice lacking p27 display an increase in overall body size due to a general increase in cell number of most tissues, but otherwise show no gross morphologic abnormalities (Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). Importantly, p27-deficient mice develop spontaneous benign lesions only in the intermediate lobe of the pituitary gland, a portion of the pituitary absent in humans (Nakayama et al., 1996). Thus, loss of p27 is not deleterious for normal cell cycle progression. Nevertheless, mutations in p27 are extremely rare in cancer. Although overall expression of p27 is often reduced with tumor progression, in almost all tumors a detectable heterogeneous expression ensures that p27 is present throughout and at high levels in at least some tumor cells (Catzavelos et al., 1997; Loda et al., 1997; Porter et al., 1997; Tan et al., 1997). Retention of p27 in tumors, and lack of a correlation with mitotic index (Catzavelos et al., 1997; Loda et al., 1997) suggests that this CKI may have other functions and may, for example, act as a survival factor for tumor cells. Thus, tumor cells with high p27 may be more equipped to resist anticancer therapy, and to cope with a changing and unpredictable microenvironment. It is noteworthy that the related CKI p21 has been shown to protect cells from apoptosis (Wang and Walsh, 1996; Waldman et al., 1996). However, the precise mechanisms by which p21 (and possibly p27) prevent apoptosis are at present unclear.

Assuming p27 is a tumor cell survival factor then antagonists of its function could potentially be useful chemosensitizers in conjunction with conventional anticancer therapy. If normal tissues retain intact inhibitors of cell cycle such as p15^INK4b and p16^INK4a (which are often mutated in tumors), such CKIs may ensure quiescence of normal tissues while p27 is being therapeutically downregulated, thus minimizing normal tissue toxicity. Although speculative, there
is some experimental support for this possibility. Studies such as ours suggest that tumor cells depend on p27 for contact-dependent growth inhibition while others have shown that p27-deficient fibroblasts are capable of contact inhibition (Nakayama et al., 1996). Although this could reflect differences in the regulation of contact inhibition of different cell types, p27 levels still increase and can inhibit cyclin E associated kinase activity in contact inhibited fibroblasts (Hengst et al., 1994; Vlach, 1996). An alternative hypothesis is that p27 regulates cell growth primarily during development, but upon differentiation other functionally redundant cell cycle inhibitors are turned on (in addition to p27) by cell-cell contact. For example, recent data suggest that in addition to p27, p16 may also promote contact inhibition of growth (Higashi et al., 1997). Such redundancy could act as a safeguard to ensure the maintenance of contact inhibition in differentiated cells. Importantly, p16 may also regulate senescence of certain normal cell types (Alcorta et al., 1996; Zindy et al., 1997; Palmero et al., 1997, Uhrbom et al., 1997). Tumor cells often lose p16 function which may enable them to partially overcome both contact inhibition and cell senescence. Thus, depleting p27 from p16 negative (-/-) tumor cells may have a much more pronounced effect on contact inhibition than in the case of normal cells which are p16 positive. If so, then combining antagonists of p27 with cytotoxic agents may promote inappropriate entry into S-phase before genotoxic damage can be repaired thus making tumor cells more sensitive to anticancer therapy. In contrast, normal cells may be somewhat protected due to the maintenance of intact cell cycle inhibitors such as p16. In vivo experiments are needed in order to address the impact of p27 on drug resistance in vivo (see section 5.2.3)
5.1.6 E-cadherin-dependent growth suppression is linked to elevated p27 levels

The exact mechanism responsible for adhesion of EMT-6 cells is unknown. It is possible, however, that various adhesion mechanisms have similar effects on cell proliferation. This hypothesis is supported by the fact that many normal cells types display contact-inhibition of growth and tumor cells often display a similar phenomenon referred to as ‘contact-dependent growth inhibition’ (St. Croix et. al., 1996a). In chapter 4, this hypothesis was tested directly by transfecting the homophilic cell adhesion molecule E-cadherin into EMT-6 tumor cells which lacked E-cadherin expression. Indeed, by disrupting adhesion of EMT-6/E-cadherin transfectants with E-cadherin neutralizing antibodies this adhesion mechanism was shown to be responsible for holding these cells together. Thus, on the same genetic background (ie. EMT-6 cells) the effects of two distinct intercellular adhesion mechanisms could be compared. The first is calcium independent, hyaluronidase sensitive, and can be activated by exposure to chemotherapeutic agents. The second is calcium-dependent and mediated by E-cadherin. Adhesion mediated by either mechanism prevented cell proliferation, and this was associated with an increase in the level of p27. Subsequently, it was shown that human breast, colon and lung carcinoma cell lines which retain endogenous E-cadherin expression can also be stimulated to grow, similar to EMT-6/E-cadherin transfectants, by adding E-cadherin neutralizing antibodies to three-dimensional cultures. Thus loss of E-cadherin in tumors may stimulate growth through downregulation of p27.

5.1.7 Signalling Between E-cadherin and p27: A Role for Classical Mitogenic Pathways

Although the phenomenon of contact inhibition is widely appreciated, only recently has it become clear that cell adhesion molecules are required for this process (Aoki et al., 1991; Kunath
et al., 1995; Hsieh et al., 1995; Sporns et al., 1995; Kandikonda et al., 1996; Takahashi and Suzuki, 1996; Caveda et al., 1996). The mechanisms by which cell adhesion molecules regulate growth is unclear. The studies of chapter 4 help to unravel the pathway by which E-cadherin signals to the nucleus and ultimately affects growth and levels of p27. For these studies, the human HT29 colon carcinoma cell line was utilized because it expresses high levels of endogenous E-cadherin and continues to grow, albeit more slowly, when cultured in serum-free medium. Interestingly, under serum free conditions, E-cadherin neutralizing antibodies were unable to stimulate growth of HT29 cells even though intercellular adhesion was completely prevented. Likewise, under these conditions p27 levels remained high and were unaffected by antibody treatment. Thus, cell-cell contact regulates p27 levels indirectly by preventing the activity of growth factors in the medium. When optimal concentrations of either FCS or TGF-α were added to three-dimensional cultures a mild growth stimulation (2- and 3-fold respectively) was observed. However, when either FCS or TGF-α was added in combination with E-cadherin neutralizing antibody, a synergistic effect on growth was observed (5- and 10-fold respectively). Correspondingly, levels of p27 were lowest when mitogens and E-cadherin antibodies were added simultaneously. Importantly, we could completely abolish the effect of TGF-α on both p27 levels and growth by adding to cell cultures C225 neutralizing antibody against epidermal growth factor receptor (EGFR). These results demonstrate that mitogens including TGF-α, are essential for maintaining both a high growth fraction and low p27 levels in the presence of E-cadherin antibodies.

5.1.8 A Model Describing the Role of p27 in E-cadherin-Dependent Growth Suppression

Based on the studies of chapter 4 and those reported by others, the following model was
constructed to help explain the effect of E-cadherin on p27 levels (see figure 5.3). One mechanism by which cell-cell adhesion may suppress growth is by inhibiting mitogen activated pathways initiated from receptor tyrosine kinases (RTKs) such as the EGFR. Activation of RTKs by growth factors has been shown to decrease p27 protein levels (Peng et al., 1996) possibly due to enhanced ubiquitin-mediated degradation of p27 (Pagano et al., 1995), but other transcriptional and translational mechanisms may be involved (Millard et al., 1997). Cadherin-catenin complexes have been found by co-immunoprecipitation studies to interact with RTKs (Hoschuetzky et al., 1994; Shibata et al., 1996), and these molecules co-localize at sites of cell-cell contact (Roy et al., 1989; Fukuyama and Shimizu, 1991; Crepaldi et al., 1994). Although growth factors rapidly induce phosphorylation of RTKs in sparse monolayer cultures, RTKs are hypophosphorylated in contact inhibited cells even in the presence of excess ligand (Takahasi and Suzuki, 1996; Mansbridge et al., 1992; Sorby and Ostman, 1996). Phosphatase inhibitors rescue growth factor induced RTK phosphorylation suggesting that phosphatases are also involved in contact-dependent growth inhibition (Mansbridge et al., 1992; Sorby and Ostman, 1996). Receptor protein tyrosine phosphatases (RPTPs) that span the extracellular membrane are upregulated and activated by cell-cell contact (Pallen and Tong, 1991; Ostman et al., 1994; Gebbink et al., 1995; Gaits et al., 1995) and have been shown to co-localize with cadherin-catenin complexes (Wang et al., 1996; Kypta et al., 1996; Balsamo et al., 1996). Tight adhesion mediated by E-cadherin or other adhesion mechanisms may strengthen interactions between adjacent RPTPs ensuring high continuous phosphatase activity.

As previously discussed, a limited number of studies have shown that E-cadherin neutralizing antibodies can prevent contact-dependent growth inhibition (Miyaki et al. 1995; Watabe et al. 1994).
Figure 5.3 Role of p27 in contact dependent growth inhibition. E-cadherin-mediated adhesion may prevent growth by inhibiting the signalling of growth factor receptors present on the surface of cells. This may occur through contact-dependent activation of receptor protein tyrosine phosphatases. Cells may also be able to sense changes in actin cytoskeleton organization, and these changes may lead to altered regulation of p27 (see text for details). GF-growth factor; β-β-catenin; γ-γ-catenin; α-α-catenin; RTK- receptor tyrosine kinase; RPTP- receptor protein tyrosine phosphatase.
E-cadherin neutralizing antibodies have also been shown to promote growth factor induced phosphorylation of RTKs even at high cell densities (Takahashi and Suzuki, 1996). In addition to inactivating RTKs, phosphatases may also promote E-cadherin-mediated adhesion (Kypta et al., 1996; Balsamo et al., 1996) by dephosphorylating β-catenin since tyrosine phosphorylated β-catenin is not observed in cadherin-catenin complexes bound to the actin cytoskeleton (Hoschuetzky et al., 1994). Conversely, RTKs can phosphorylate β-catenin, and this may promote destabilisation of E-cadherin-mediated adhesion (Shibata et al., 1996; Shibamoto et al., 1994). Although many of the studies used to generate this model were conducted on tumor cells, some were performed on immortalized epithelial cell lines in culture (Takahashi and Suzuki, 1996; Kandikonda et al., 1996). This model may, therefore, also have relevance for understanding contact inhibition of normal cells.

Based on experiments from chapter 3, there are at least two independent adhesion mechanisms capable of regulating p27; one dependent on E-cadherin and the other on HA. In addition to adhesion, other architectural mechanisms are also likely to regulate p27 protein levels. When cells were transferred from monolayer tissue culture dishes to suspension but were prevented from adhering to one another with hyaluronidase, p27 levels increase significantly (see chapter 3). Thus, changes in cell shape and/or cell-extracellular matrix interactions may also regulate p27 protein levels. Another study indicates that RhoA and RhoB, small GTPase’s known to regulate the cytoskeleton, may also affect p27 protein levels (Hirai et al., 1997). Taken together, such studies suggest that cells may be able to sense changes in actin cytoskeleton organization, and this may also be involved in growth regulation through p27.
5.1.9 Does E-cadherin Regulate Drug Resistance?

Because E-cadherin-dependent adhesion regulates p27 levels in a manner similar to HA-dependent adhesion, E-cadherin may be a physiological regulator of intrinsic drug resistance of carcinoma cells which fail to lose E-cadherin activity. Indeed, one correlative study suggests that this may be the case (Dimanche-Boitrel et al., 1994). E-cadherin may also play a role in acquired resistance to anti-cancer agents (Bracke et al., 1994; Skoudy et al., 1996) although other adhesion mechanisms, as in the case of our EMT-6 drug resistant variants, are likely to be involved.

If E-cadherin does regulate drug resistance, there are several important ramifications. For example, it may be possible to predict which carcinomas will respond to therapy based on whether or not E-cadherin is present in tumor biopsies. In other words, a lower level of E-cadherin, which in non-treated patients is an unfavorable prognostic indicator (Siitonen et al., 1996), may actually predict a better response (at least initially) to cytotoxic therapy. If E-cadherin can regulate resistance only through its effects on cell cycle, then its usefulness may be limited when compared to currently used prognostic indicators such as S-phase fraction. On the other hand, if E-cadherin regulates drug resistance through other mechanisms (for example, by preventing drug induced apoptosis), then monitoring its levels could have prognostic potential. Such information could help oncologists to determine what type of therapy to give individual patients and when to give cancer patients a more aggressive anticancer treatment. E-cadherin status may also enable oncologists to predict which tumors will respond most effectively to antibodies such as 4D5 and C225 currently being used in clinical trials to target the RTKs erb-B2 and EGFR, respectively (Baselga and Mendelsohn, 1994; Mendelsohn and Fan, 1997). Regulation of p27 by E-cadherin could also help to explain why these molecules are often co-ordinately lost in certain tumors (for example in high grade invasive but not
low grade ductal breast carcinomas) (Catzavelos et al., 1997; Tan et al., 1997; Siitonen et al., 1996; Berx et al., 1996).

5.1.10 Anti-Adhesives as Sensitizers for Cytotoxic Therapy

The use of anti-adhesives in cancer therapy has been studied almost exclusively in the context of invasion, angiogenesis, and metastasis (Platt and Raz, 1992; Glinsky et al., 1996). However, as our studies suggest, anti-adhesive agents may also be useful for the treatment of cancer when combined appropriately with other conventional or experimental anti-cancer drugs (Kerbel et al., 1995b). Anti-adhesives may be particularly efficacious in the treatment of malignant ascites (Schneider, 1994). Unfortunately, in some cases E-cadherin expression is reduced or absent in ascites of ovarian or colorectal carcinoma, even though these cells typically aggregate (Veatch et al., 1994; Kitsuki et al., 1995, Darai et al., 1997). Likewise, when E-cadherin neutralizing antibodies were tested on a panel of ovarian cancer cell lines, intercellular adhesion was only prevented in two out of eight cases (unpublished observations). Thus, E-cadherin neutralizing antibodies may be ineffective at disrupting and chemosensitising some ovarian ascites tumors. However, in such cases there may be a silver lining for this type of an approach if the mechanism holding tumor cells together is different from that found on adjacent normal tissues. For example, one recent report suggests that CEA is primarily responsible for intercellular adhesion of ascites cells from colorectal carcinomas (Kitsuki et al., 1995). If so, then antagonists of CEA may chemosensitize colorectal ascites tumors without affecting the sensitivity of normal adjacent cells which lack CEA expression. As an alternative approach, it may be more practical to target critical downstream targets of cell-cell adhesion. For example, antagonists of CKIs such as p27 may represent a novel class of
chemosensitizers useful in augmenting anticancer therapy (see section 5.1.5).

5.1.11 ‘Multimodality Resistance’

While the literature is not yet extensive, the results summarized in this thesis point to a possible critical role for intercellular adhesion in the resistance of solid tumors to conventional cytotoxic anticancer drugs. Much of what is known in this new area of research stems from the study of three dimensional cultures of tumors, e.g. multicellular spheroids. This alone raises two important points. First, because of the overwhelming use of two-dimensional (monolayer) cell culture systems to study drug resistance in cancer, the impact of cell adhesion in drug resistance has not been appreciated or recognized. Second, it is well known that tumor spheroids express what might be called "multimodality resistance", that is, they not only express intrinsic resistance to a diverse class of cytotoxic chemotherapeutic drugs, but also to such agents as ionizing radiation (Sutherland, 1988; Olive and Durand, 1994), immunotoxins (Chignola et al., 1995), activated killer cells (Ochalek and Kleist, 1994), complement mediated cell lysis (Bjorge et al., 1997), cytostatic cytokines (Gorlach et al., 1994) and hyperthermia (Sutherland, 1988; Olive and Durand, 1994). The mechanisms underlying this pleiotropic resistance are probably equally diverse and may include reduced penetration of the therapeutic agent (Ochalek and Kleist, 1994) and resistance to apoptosis (Helmlinger et al., 1997). It follows that the concept of using anti-adhesive agents to reverse multicellular resistance to cytotoxic drugs may be applicable to many types of anti-cancer agents which are subject to multicellular resistance.
5.2 Future Directions

The following section outlines directions for future work inspired by the studies discussed in the preceding chapters of this thesis. Although many questions have been raised by the previous work, the focus in this section is on those experiments considered to be the most interesting and important by the author. Obviously, such experiments will reflect some degree of personal bias. Perhaps only time will tell which avenues will be the most fruitful.

While most of the experiments described in this section are still in the contemplative stage, a few of the studies described have been initiated and are therefore outlined in the context of preliminary results. Results from prospective future work should further illuminate the impact of cell adhesion molecules on growth regulation and multicellular drug resistance. It is hoped that such work will also lead ultimately to the development of better anticancer treatment strategies.

5.2.1 Inactivation of Growth Factor Receptors by E-cadherin

The importance of growth factors and growth factor receptors in mitogenesis is well established. The impact of cell-cell adhesion receptors on cell cycle progression, on the other hand, is only beginning to be appreciated. A careful review of the literature in this area suggests that one possible mechanism by which cell adhesion can retard growth by inhibiting the activity of growth factor receptors. In particular, cell-cell contact has been shown to inhibit ligand-induced receptor tyrosine kinase activity of the EGFR or erbB2 (Lichtner and Schirrmacher, 1990; Mansbridge et al., 1992). One study demonstrated that anti-E-cadherin neutralizing antibodies could restore phosphorylation of EGFR in response to EGF (Takahashi and Suzuki, 1996). Exactly how E-cadherin is able to inactivate RTKs, however, remains unclear. Thus, studies of the modulation of
growth factor receptor activity by adhesion molecules represents one potentially interesting area of research for future studies.

Clues as to how E-cadherin may inactivate RTKs comes from studies on the phosphorylation of β-catenin. An important initial study demonstrated that phosphorylated β-catenin is not observed in cadherin-catenin complexes bound to the actin cytoskeleton (Hoschuetzky et al., 1994). Furthermore, β-catenin could be co-immunoprecipitated with the EGFR suggesting that this RTK may be responsible for phosphorylation of β-catenin. One recent study provides further support for the hypothesis that RTKs are able to directly phosphorylate β-catenin (Shibata et al., 1996). However, the impact of β-catenin phosphorylation on intercellular adhesion, if any, is unknown. Although a number of studies have documented a correlation between β-catenin phosphorylation and reduced intercellular adhesion, there is no direct evidence linking these two phenotypes. Furthermore, one study demonstrated that β-catenin phosphorylation is not required to shift cadherin-mediated adhesion from the strong to the weak state in V-src transformed MDCK cells (Takeda et al., 1995).

While RTK stimulation may destabilize cadherin-mediated adhesion through phosphorylation of β-catenin, mechanisms by which E-cadherin mediated adhesion suppresses RTK activity have not been addressed. For example, an association between β-catenin and RTKs may be necessary for E-cadherin-dependent RTK inactivation. The TMK-1 human gastric cancer cell line which expresses wild-type E-cadherin has been engineered to express an N-terminal deleted β-catenin. This mutated β-catenin binds to the EGFR and can act as a dominant negative to inhibit the phosphorylation of wild-type β-catenin (Shibata et al, 1996). Although invasion and tumorigenicity of the transfectants
was suppressed, alterations in intercellular adhesion were not assessed. An analysis of RTK activity in these cells may enable determination of whether β-catenin is necessary for inactivation of RTKs in response to E-cadherin-mediated adhesion.

An alternative hypothesis is that adhesion mediated by E-cadherin results in RTK inactivation through upregulation of phosphatase activity. In support of this idea, a number of studies have shown an upregulation of membrane bound phosphatase activity in response to cell-cell contact, and RPTPs colocalize with cadherins at sites of cell-cell contact (Balsamo et al., 1996; Kypta et al., 1996; Wang et al., 1996). Furthermore, treatment of cells with phosphatase inhibitors such as orthovanadate has been reported to counteract contact-dependent RTK inhibition (Mansbridge et al., 1992; Sorby and Ostman, 1996). If this also holds true for cell lines such as HT29, then an analysis into the expression levels of various known RPTPs in response to E-cadherin mediated adhesion could be undertaken. In general RPTPs have a rather broad substrate specificity and any of a number of known RPTPs could potentially be involved in dephosphorylating RTKs. However, substrate specificity may be directed by the formation of functional adherens junctions which contain actin-bound cadherin-catenin complexes and may serve as docking sites for both RPTPs and RTKs. In support of that idea, an RPTP called LAR has recently been shown to interact directly with β-catenin (Kypta et al., 1996; Aicher et al., 1997). As well, a cytoplasmic PTP has been shown to interact with the cytoplasmic tail of N-cadherin (Balsamo et al., 1996). The involvement of such phosphatases in E-cadherin-dependent growth inhibition has not been addressed. It seems likely that RPTPs will play a central role in the process of contact-inhibition and further studies into area are needed.

The fact that E-cadherin inhibited mitogenic stimulation by both TGF-α and FCS in HT29
cells (chapter 4) raises the hypothesis that E-cadherin-mediated adhesion may inactivate several mitogenic pathways. This hypothesis is supported by the fact that β-catenin can interact with several RTKs including EGFR, ErbB-2 and possibly c-met (Hoschuetzky et al., 1994; Kanai et al., 1995; Shibata et al., 1996). Furthermore, recent preliminary experiments have demonstrated that E-cadherin-mediated adhesion can inhibit growth stimulation of MCF-7 cells in response to insulin-like growth factor I (IGF-I). The IGF-I receptor is also a receptor tyrosine kinase family member. Analogous interactions may also occur in different cell types. For example, vascular endothelial growth factor (VEGF) is responsible for stimulation of another RTK, the VEGF receptor, which is specifically expressed on endothelial cells. Recently, vascular-endothelial cadherin (VE-cadherin) has been shown to be necessary for contact-inhibition of endothelial cells (Caveda et al., 1996). Further experiments are needed to determine whether adhesion mediated by VE-cadherin inhibits growth by repressing VEGFR tyrosine kinase activity following ligand stimulation. Such experiments will help to determine the specificity of cadherin-mediated RTK inactivation.

5.2.2 Does p27 Regulate Drug Resistance In Vivo?

The results of chapter 3 demonstrate that p27 can regulate drug resistance in vitro. However, further experiments are needed in order to address the impact of p27 on drug resistance in vivo, and determine whether or not antagonists of its function will be effective chemosensitizers. As a first approach, it would be useful to compare p27 staining of immunohistochemical sections of tumors before and following therapy. To date, such a study has not been published. However, immunohistochemical sections from non-treated patients stained for p27 demonstrate that its levels are very heterogenous (Catzavelos et al., 1996, Loda et al., 1996). It is possible, therefore, that a
selection occurs for ‘high p27 expressors’ following anticancer therapy. Because p27 levels can fluctuate significantly between tumors, the most effective way to test this hypothesis would be to obtain paired samples from the same patient. As well, timing of biopsy removal following therapy may be of critical importance in determining whether or not elevated levels of p27 are observed. This may be particularly crucial if resistance is of the intrinsic type, since selection for tumors cells harbouring high p27 levels may occur only transiently following therapy. If so, p27 levels may gradually decrease following therapy and eventually become heterogenous again as the tumor cell population resumes growth and becomes asynchronous. However, if resistance is of the acquired type and is maintained in a stable manner, then timing of biopsy removal may be less of a concern.

If immunohistochemical staining supports a role for p27 in resistance, then a logical next step would be to antagonize p27 levels or activity in vivo, treat cells with anticancer agents and monitor chemo-responsiveness of both tumor cells and host cells. Although a small molecular weight protein antagonist of p27 may be preferable, such an agent does not yet exist. Instead, it may be more practical, at least initially, to employ an antisense approach using reagents such as the C5-propyne-substituted p27 antisense oligonucleotides previously employed in vitro (see chapter 3). In this regard, preliminary in vivo studies have shown that fluorescent-tagged C5-propyne-modified oligonucleotides (containing non-specific sequences) are effectively taken up by EMT-6 cells growing as an ascites tumor. Given the effectiveness of C5-propyne modified oligonucleotides on EMT-6 cells in vitro, this may be a good model to begin assessing the potential of downregulating p27 in vivo for augmenting anticancer therapy.
5.2.3 The Impact of E-cadherin on Drug Resistance of Tumor Cells

It is unclear whether adhesion mediated by E-cadherin can regulate resistance of tumor cells to anticancer agents. However, this seems likely given that E-cadherin can regulate p27 levels and an increase in p27 in response to HA-dependent adhesion was shown to be necessary for drug resistance (chapter 3). The models that we have employed for our previous studies, especially the HT29 colorectal cancer cell line, should be useful to address this question in future studies. As previously discussed, HT29 cells express endogenous E-cadherin and can be dispersed with E-cadherin neutralizing antibodies. However, growth is only stimulated if mitogens (such as TGF-α or FCS) are also added to the culture medium. Thus, by growing HT29 cells in mitogen-free medium, it should be possible to test the effect of adhesion, in the absence of a growth suppressive effect, on resistance to anticancer therapy. Hopefully, such experiments will enable determination of whether the effect of adhesion on drug resistance is completely dependent, partially dependent, or independent of increased cell proliferation. The reason this question remains unresolved is that, despite much effort, models used in previous studies have not allowed separation of the adhesion phenotype from the growth phenotype. For example, EMT-6 cells were found to depend upon serum for their survival precluding the removal of FCS from the culture medium. Furthermore, when tightly-adherent EMT-6 cells were stimulated to grow with antisense oligonucleotides against p27, cells lost their adhesive phenotype. Thus, it is theoretically possible that depletion of p27 by antisense oligonucleotide treatment sensitized tumor cells to cytotoxic therapy indirectly through effects on intercellular adhesion rather than on cell cycle changes *per se*. Because induction of p27 in H/MT27 cells causes growth suppression but does not result in increased intercellular adhesion in the presence of excess E-cadherin antibodies, this system could also be used to determine the
relative contribution of growth and adhesion to the resistance phenotype. In this case cells could be cultured in the presence of FCS.

5.2.4 Anti-Adhesives as Chemosensitizers in the Treatment of Ovarian Ascites Tumors

Ovarian cancer, at the later stages of tumor progression, frequently spreads into the peritoneal cavity where it often grows as mixture of single cells, free floating cellular clumps and tumor nodules attached to the peritoneal wall. Advanced-stage disease is often associated with a build up of abdominal fluid, known as ascites. Unfortunately, because the earlier stages of the disease are usually asymptomatic, approximately 75% of ovarian cancer patients display advanced disease at diagnosis (Perez et al., 1993). The standard treatment for advanced-stage ovarian cancer is cytoreductive surgery followed by administration of combination chemotherapy. Previously, standard treatment protocols most often employed either cisplatin or carboplatin in combination with cyclophosphamide. More recently, treatment regimens which include either Taxol/cisplatin or Taxol/carboplatin have proven more effective (Ozols and Vermorken, 1997). Chemotherapy results in a complete clinical response rate in 40-60% of patients with advanced-stage disease (Johnson et al., 1993). However, the primary reason for treatment failure is the emergence of drug resistance in the tumor cell population. Upon second-line treatment with Taxol/cisplatin or Taxol/carboplatin (or other unrelated cytotoxic agents), the tumor usually shows little or no response suggesting the emergence of a multiple drug resistance phenotype. Indeed, exposure to cisplatin can result in overexpression of P-glycoprotein even though cisplatin is not a substrate of the drug efflux pump (Yang and Page, 1995). The overall 5-year survival rate for advanced-stage ovarian cancer patients is approximately 20% (Perez et al., 1993). However, late relapses are common and virtually all
patients with advanced-stage tumors resistant to second-line chemotherapy will succumb to their disease. Thus, ovarian cancer, which afflicts one of 70 North American women, is considered a deadly disease (Ozols and Vermorken, 1997; Nahhas, 1997).

Ascites tumors are, by definition, associated with an accumulation of excess fluid in the peritoneal cavity. One retrospective study demonstrated that if patients with advanced ovarian carcinoma were separated into two groups, those with ascites and those without ascites, the median 5-year survival of the former was 5% while that of the latter was 45% (Puls et al., 1996). The median survival of patients with ovarian ascites is approximately 6 months (Dauplat et al., 1987). Although spread of advanced disease is frequently only detected in the abdominal cavity or regional lymph nodes, metastasis have been found in several organs, such as the liver, lung, and distant lymph nodes (Dauplat et al., 1987). Thus, successful eradication of drug resistant tumors which appear to be confined to the abdominal cavity, would not guarantee cure of the disease. For example, clinically undetectable distant metastasis which otherwise would have gone undetected may begin to emerge. Nevertheless, successful eradication of intra-abdominal ovarian tumors is expected to prolong patient survival time, and may result in an increased number of cures.

The fact that advanced ovarian carcinoma frequently appears to be confined to the peritoneal cavity makes this an attractive model for studying the potential of using anti-adhesives as chemosensitizers in vivo. Further incentive for such experiments derives from in vitro studies which demonstrate that ovarian cancer cells cultured as spheroids are relatively resistant to anticancer agents such as Taxol when compared with dispersed monolayer cultures (Frankel et al., 1997). Although the mechanisms governing resistance of spheroids to Taxol is unknown, given the cell cycle specificity of this agent, resistance may have a kinetic basis. Limited drug diffusion is also
likely to contribute to resistance due to the drug's bulky structure and high molecular weight.

Regardless of the resistance mechanism(s), anti-adhesives may be useful chemosensitizers (Kerbel et al., 1995; see also chapter 2). It is noteworthy that in patients with advanced ovarian carcinoma, the maximum dimension of residual tumor masses is highly predictive of treatment response (Gallion et al., 1987; Piver et al., 1994; Schneider, 1994). Thus, response rates are 40% to 50% in patients with residual tumor nodules less than 0.5 cm compared with less than 15% in patients with larger tumors (Schneider, 1994). Furthermore, the favourable pharmacokinetics that are achievable with intraperitoneal drug administration (see section 1.1) have been most effective in patients with residual tumor masses less than 1-2 cm in greatest dimension (Alberts et al., 1996; Kirmani et al., 1991). It has been hypothesised that residual tumor nodules with a larger size are less responsive due to insufficient drug diffusion into the centre of the tumor mass (Markman et al., 1995; Alberts et al., 1996). Indeed, following intraperitoneal drug administration limited penetration of cisplatin, carboplatin and Adriamycin into tumor nodules has been demonstrated in rodents (Los et al., 1991; los et al., 1989; Ozols et al., 1979).

Given the ability of anti-E-cadherin antibodies to completely abolish intercellular adhesion of several carcinoma cell lines, this agent may be the most useful anti-adhesive to begin chemosensitization studies of ovarian cancer cells. Importantly, anti-E-cadherin neutralizing antibodies are highly effective at disrupting adhesion not only when added to three-dimensional cultures at the time of cell plating, but also when added to preformed, one week old spheroids (unpublished observations). Thus, anti-E-cadherin antibodies may be able to disperse established ascites tumors which are already growing as free-floating clumps, or tumor nodules attached to the peritoneum.
As previously mentioned, some studies (Veatch et al., 1994; Darai et al., 1997), but not all (Sundfeldt et al., 1997), have documented a loss of E-cadherin expression in ovarian cancer during the later stages of tumor progression. In normal ovaries E-cadherin expression is undetectable except in the inclusion cysts. In benign ovarian tumors, however, E-cadherin appears to be uniformly expressed (Sundfeldt et al., 1997; Darai et al., 1997). Thus, E-cadherin levels may increase early following transformation and subsequently, as the cells become more invasive, decrease or become heterogeneous in some, but not in all ovarian carcinomas. Decreased levels of E-cadherin have been postulated to facilitate tumor cell detachment from the solid tumor masses (Veatch et al., 1994).

Although ovarian cancer cells which lack functional E-cadherin typically retain some ability to aggregate, the mechanism(s) responsible for intercellular adhesion in these cases are unclear. Because some advanced ovarian carcinomas fail to lose E-cadherin expression, in these carcinomas E-cadherin may be functional. In support of this idea, when anti-E-cadherin neutralizing antibodies were tested on a panel of eight ovarian carcinoma cell lines, two cell lines, SW626 and CAOV3, were completely dispersed (unpublished observations). Given the well defined adhesion mechanism of these two cell lines, they are currently the most suitable to begin studies on the effect of adhesion on resistance of ovarian ascites tumors in vivo. Depending on the results, such studies could provide further incentive for uncovering other mechanisms of adhesion responsible for holding ovarian cancer cells together.

Before beginning in vivo experiments which are often labour intensive and costly, first in vitro experiments would be conducted. Using the SW626 and CAOV3 cell lines, the effect of E-cadherin-mediated adhesion on resistance to cytotoxic agents in three-dimensional cultures would
be examined. For measuring drug resistance, clonogenic assays, which are often considered the gold standard amongst in vitro drug resistance assays, would be employed. E-cadherin-mediated adhesion in three-dimensional cultures would be inhibited with the SHE78-7 anti-E-cadherin antibody which disrupts adhesion of these cell lines at low (~1 μg/ml) concentrations (unpublished observations). As a control, monolayers would also be treated with anti-E-cadherin antibodies and then assayed for resistance. For initial experiments Taxol would be the drug of choice for several reasons. First, clinical studies have proven its efficacy against ovarian carcinoma. Cell culture studies have also shown that exposure of ovarian cancer cells to increasing concentrations of Taxol results in progressively higher levels of cell kill. Also, Taxol is a highly cycle-specific agent which could enhance toxicity in combination with anti-adhesives that stimulate tumor cell growth. Following intraperitoneal injection Taxol displays the most favourable peritoneal fluid to plasma drug ratios of any anticancer agent analysed (see table 1.1). Finally, Taxol has been shown to be more effective on ovarian cancer cells cultured as monolayers when compared with spheroids (Frankel et al., 1997).

If E-cadherin-mediated adhesion is shown to regulate Taxol resistance of ovarian carcinoma cell lines in vitro, then the effect of E-cadherin on in vivo resistance of ovarian carcinoma cell lines would be addressed. For this purpose, a survival assay would be designed similar to the one described in chapter 2. To begin, SW626 or CAOV3 cells would be injected intraperitoneally into nude mice and subsequently examined at various days following inoculation for the formation of tumor clumps. At the appropriate time point (i.e. once tumor nodules or free-floating aggregates have formed), various concentrations of SHE78-7 antibody would be administered and the effect on adhesion monitored. If E-cadherin-mediated intercellular adhesion can be disrupted in vivo, then a survival assay would be designed. Taxol would be the drug of choice for these experiments,
provided anti-E-cadherin antibodies sensitize cells to Taxol in vitro.

5.2.5 Antiadhesives as Chemosensitizers for Chemosensitizers

Previous studies have shown that reversal of P-glycoprotein-mediated multidrug resistance using chemosensitizers such as verapamil is much harder to achieve in spheroids compared to monolayer cell cultures (Sakata et al., 1994). Indeed, the relative resistance of spheroids may help to explain the rather disappointing results of clinical trials of such agents in patients with multidrug resistant solid tumors (Houghton and Kaye, 1994). Conversely, absent or low levels of intercellular adhesion may help to explain the promising results of clinical trials aimed at reversing multiple drug resistance in haematological malignancies (Dalton, 1994). Because of the anti-adhesive effect of hyaluronidase on murine EMT-6 cells, we asked whether this agent could increase the chemosensitizing effect of the cyclosporin analogue known as PSC-833 which is a strong antagonist of P-glycoprotein in monolayer cell culture. Similar to studies by Dr. Robert Sutherland and colleagues (Sakata et al., 1994), preliminary studies have shown that EMT-6 variants selected for high levels of P-glycoprotein and a multidrug resistant phenotype (Twentyman et al., 1990) were effectively chemosensitized in monolayer but not spheroid culture. However, if the spheroids were disaggregated with hyaluronidase, the efficacy of PSC-833 was increased by at least three orders of magnitude (St. Croix and Kerbel, 1997). This raises the possibility of using anti-adhesives as "chemosensitizers for chemosensitizers".

More than half of ovarian carcinomas have been shown to express high levels of P-glycoprotein either endogenously or in response to chemotherapy (Khalifa et al., 1997). Furthermore, following chemotherapy patients with P-glycoprotein positive tumors were at a three
times greater risk of dying within two years than patients with P-glycoprotein negative tumors (Khalifa et al., 1997). While clinical trials are underway, at present it is unclear whether reversal agents such as cyclosporin A will be useful in sensitizing ovarian carcinomas to chemotherapy. Results of a recent phase I clinical trial demonstrated that cyclosporin A injected intraperitoneally displayed very favourable pharmacokinetics, presumably due to its high molecular weight and metabolic inactivation in the liver (Chambers et al., 1997). Thus, peritoneal fluid to blood ratios of 1000:1 were observed. However, because of its high molecular weight, cyclosporin A may also penetrate large tumor nodules poorly. As well, increased cell-cell adhesion may also result in a higher intrinsic resistance to P-glycoprotein reversal agents (Sakata et al., 1994).

Regardless of the reasons for resistance, preliminary in vitro studies with EMT-6 suggest that the efficacy of P-glycoprotein antagonists in vivo may be substantially enhanced by the use of anti-adhesives. To test this idea in an ovarian cancer model, the SW626 and CAOV3 cell lines would be transfected with a P-glycoprotein expression vector. Before beginning in vivo studies, in vitro studies would be undertaken to ensure that anti-E-cadherin antibodies can sensitize cells to P-glycoprotein antagonists. SHE78-8 anti-E-cadherin neutralizing antibodies would be used to disrupt adhesion, and PSC-833 would be used to antagonize P-glycoprotein. Because Taxol is a P-glycoprotein substrate, this drug would be used for in vitro drug sensitivity assays. Provided E-cadherin does regulate the ability of P-glycoprotein antagonists to function in vitro, in vivo survival assays would be designed to address whether E-cadherin has the same effect on ovarian ascites tumors.
5.3 Overall Summary

The studies described in this thesis originated from an investigation of the nature of multicellular drug resistance at both the cellular and molecular level. The rationale for undertaking these studies was to understand a mechanism of resistance which may help explain the basis of clinical drug resistance. A better knowledge of relevant drug resistance mechanisms should, in turn, lead to the development of novel, hopefully more effective anticancer strategies.

Initial studies demonstrated that an increase in intercellular adhesion was sufficient for multicellular drug resistance and that growth inhibition by cell-cell contact was associated with this type of resistance. Subsequently, the cyclin-dependent kinase inhibitor p27 was shown to be important for growth inhibition, and pharmacologic depletion of p27 restored drug sensitivity to tumor cells. Thus, p27 provides one possible molecular target for sensitizing tumor cells to cytotoxic therapy. Because p27 is rarely mutated in cancer, regulation of this CKI by intercellular adhesion may help to explain why common solid tumors, despite the presence of many mutated oncogenes and tumor suppressor genes, often contain low growth fractions in vivo.

Studies on E-cadherin were conducted to determine if a known cell-cell adhesion receptor could affect growth, as suggested by our previous studies. Indeed, it was demonstrated that both E-cadherin and HA-dependent adhesion have similar effects on both p27 and growth. Although not addressed here, based on its similarity to the HA-dependent adhesion mechanism, E-cadherin-dependent adhesion is also likely to affect drug resistance. The pathway by which E-cadherin dependent adhesion regulates growth depends on the presence of mitogens. Understanding the signaling pathways by which cell adhesion affects growth may also lead to novel therapeutic approaches for the treatment of cancer. For example, monitoring E-cadherin in tumors may help
oncologists to determine which tumors will be responsive to chemotherapy or antibodies such as 4D5 and C225. Furthermore, anti-adhesives may be particularly efficacious in the context of intraperitoneal chemotherapy for the treatment of advanced-stage ovarian carcinoma.
Chapter 6

References to the literature


