Extracellular matrix regulation of drug resistance in small-cell lung cancer

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ABSTRACT
Tumour recurrence following chemotherapy remains a major obstacle to the cure of many cancers. This is exemplified by small-cell lung cancer (SCLC). Host–tumour interactions are central to tumour survival and proliferation. We hypothesized that a factor(s) within the local environment of SCLC cells could provide a survival signal or block a death signal, thereby accounting for the protection of SCLC cells from chemotherapy-induced apoptosis. Here we review recent work undertaken in our laboratory addressing this issue. We have shown that, in vivo, SCLC cells are surrounded by an extensive stroma of extracellular matrix (ECM) at both primary and metastatic sites which contains, among other proteins, fibronectin, laminin and collagen IV. Furthermore, adhesion of SCLC cells to fibronectin, laminin and collagen IV through β1 integrins enhances tumorigenicity and confers resistance to apoptosis induced by standard chemotherapeutic agents, including etoposide, cis-platinum and adriamycin. Adhesion to ECM proteins stimulated protein tyrosine kinase (PTK) activity in both untreated and etoposide-treated cells. This effect could be completely blocked by a selective PTK inhibitor or by a function-blocking β1 integrin antibody. PTK activation was found to block chemotherapy-induced activation of the death protease caspase-3 and, hence, apoptosis. Adhesion to ECM or treatment with a PTK inhibitor did not affect etoposide inhibition of topoisomerase II. Thus adhesion to ECM through β1 integrins protects SCLC cells from chemotherapy-induced caspase-3 activation and apoptosis by activating PTK signalling downstream of DNA damage. Survival of tumour cells attached to ECM within this microenvironment could explain the local recurrence of SCLC and other tumours that is often seen clinically after chemotherapy.

INTRODUCTION
This year, lung cancer will kill 36000 people in the U.K. [1]. For many years lung cancer has been the commonest cause of cancer death in British men, and it has now overtaken breast cancer as the commonest cause of cancer death in British women. About 25% of these deaths will be due to small-cell lung cancer (SCLC), a particularly aggressive form of lung cancer characterized by the development of early and widespread metastases. If untreated, the median survival time after diagnosis of SCLC is only 5–12 weeks, depending upon stage. The major breakthrough in the management of SCLC occurred in 1969 with the finding that this tumour is particularly sensitive to chemotherapy and radiotherapy [2]. However, despite initial response rates of up to 80%,
these improvements tend to be short-lived, with a median duration of 6–8 months, such that the 5-year survival rate is only 3–8% [3]. Unfortunately, despite multiple trials examining new chemotherapy and radiotherapy regimens, this figure has remained virtually unchanged for the last 30 years. More and more it is being realized that, in order to make further progress towards the development of novel therapies for lung cancer, a better understanding of the molecular processes that regulate cellular growth and cell death (apoptosis) is required. Here we review recent work undertaken in our laboratory, which examines the mechanisms underlying resistance to chemotherapy in SCLC [4].

The two fundamental processes responsible for the highly malignant phenotype of SCLC are: (1) its propensity to metastasize early in its clinical course, and (2) its ability to become resistant to chemotherapeutic agents. Usually, SCLC is extremely sensitive to chemotherapeutic agents, so that tumours often shrink dramatically in size and may, at the macroscopic level, disappear entirely. However, following completion of treatment the tumour invariably recurs, indicating that microscopically the disease has survived (Figure 1).

The mechanisms involved in the acquisition of drug resistance by SCLC cells are still unclear. Several of the drugs used in the treatment of SCLC, such as etoposide and Adriamycin, act on the nuclear enzyme DNA topoisoamerase II by freezing an enzyme-DNA cleavable complex, thereby creating DNA breaks and eventually leading to cell death. Several cellular resistance mechanisms towards topoisoamerase II poisons have now been identified. The first mechanism, called the altered topoisoamerase II multi-drug resistance phenotype, is usually due to a down-regulation of enzyme amount, but can also be due to mutations leading to decreased drug sensitivity [5,6]. Such a mechanism has been described in an Adriamycin-resistant SCLC cell line [7]. Two well-characterized drug efflux pumps, P-glycoprotein (encoded by mdr1) and the multi-drug resistance protein (MRP; coded for by the MRP gene), also occur in both SCLC and non-small-cell lung cancer, and have been shown in transfection studies to be sufficient to confer resistance [8,9]. However, there is still controversy over the clinical importance of these resistance mechanisms [10,11].

**HYPOTHESIS**

It is now well established that many chemotherapeutic drugs, including etoposide, cis-platinum, Adriamycin and 5-fluorouracil, exert their effects by causing apoptosis [12]. Because tumour mass is a balance between cell proliferation and cell death, any factor affecting this balance will have a profound effect on tumour growth. In cancer biology it is becoming increasingly apparent that many tumour cells are able to circumvent chemotherapy-induced apoptosis. We hypothesized that, *in vivo*, some factor or factors in the local environment of the tumour...
Resistance to chemotherapy in small-cell lung cancer may be able to provide a survival signal, thereby accounting for the protection of SCLC cells from chemotherapy-induced apoptosis.

Many epithelial and endothelial cell types are dependent upon adhesion to the extracellular matrix (ECM) for their continued survival, and undergo apoptosis upon detachment from the matrix [13]. Although transformed cells are characterized by their ability to grow in the absence of contact with a solid ECM, solid tumour cells exist, in vivo, in a state of dynamic interplay between anchorage-dependence and -independence. Of note is the observation that, after chemotherapy, SCLC often recurs initially at the same sites as the original tumour. This suggests that tumour cells that are adhered to matrix may be relatively protected from chemotherapy compared with cells that are anchorage-independent. We reasoned that the extent and type of constituent proteins in the ECM might, in some way, be related to tumour survival.

**ECM composition of SCLC tumours**

Initially we analysed the extent of ECM proteins in 23 SCLC re-section specimens. We found that, in normal areas of lung, laminin and collagen IV were localized to the basement membranes of alveoli, septae, blood vessels and bronchial glands, whereas fibronectin was expressed at low levels throughout the pulmonary interstitium. However, no evidence of tenascin was seen. However, in SCLC samples, widespread staining for fibronectin, collagen IV and tenascin was seen in almost all sections examined. The proteins were expressed mainly in areas of reactive host connective tissue, either as extensive areas of scarring or as stromal bands delineating pockets of invasive tumour cells (Figure 2). Furthermore, in SCLC sections, the basement membranes of alveoli invaded by tumour cells were thickened considerably compared with those of adjacent uninvolved alveoli, and this was due to increased expression of the same three proteins (Figure 2). In addition to ECM deposition around tumour cells, it was noted that, in up to 25% of tumours, fibronectin and laminin staining was also visible within the cells themselves (Figure 2). Thus, in vivo, SCLC cells exist in an ECM-rich environment. In order to examine the physiological relevance of these findings, we have developed an in vitro SCLC model using several classical SCLC cell lines.

**SCLC cell integrin expression**

The main family of cell surface receptors that mediates cellular adhesion to ECM proteins is the integrin family. Integrins are composed of non-covalently associated $\alpha$ and $\beta$ subunits, which form heterodimeric receptor complexes. Integrins play a major role in regulating both proliferative activity and cell motility and, as a consequence, metastatic ability. The extracellular ligand-binding specificity of an integrin is generated jointly by the two subunits. For instance, fibronectin mediates cell adhesion and anchorage through several integrins, including $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$. Collagen and laminin bind predominantly via $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. It has been shown previously that the main $\beta$ integrin expressed by SCLC cells is $\beta_1$, and that the principal $\alpha$ integrins expressed are $\alpha_2$, $\alpha_3$, $\alpha_6$ and $\alpha_v$ [14,15]. Therefore SCLC cells express the appropriate integrins for adhesion to fibronectin, collagen and laminin.

**ECM proteins protect SCLC cells from chemotherapy-induced apoptosis**

In order to test the hypothesis that ECM proteins might protect SCLC cells from chemotherapy-induced apoptosis, we treated SCLC cell lines with a variety of
Figure 3 Effect of fibronectin on chemotherapy-induced apoptosis in H69 SCLC cells

SCLC cells (1 × 10⁵/ml) were seeded into 96-well plates pre-coated with fibronectin (Fn; 20 µg/ml) or 1% (w/v) BSA. After 1 h, etoposide (25 µg/ml) was added and cells were incubated further at 37 °C. After 24 h the plates were spun at 200 g for 4 min, supernatant was aspirated and apoptosis was detected using either Acridine Orange/ethidium bromide staining (left panel) or a commercial cell death ELISA kit (right panel). Values are means ± S.E.M. of three independent experiments performed in triplicate. Significance of differences: * P < 0.05 compared with cells on plastic treated with etoposide.

chemotherapeutic agents, including etoposide, adriamycin and cyclophosphamide, in the presence and absence of various ECM proteins. We found that cells that adhered to fibronectin, laminin or collagen IV were markedly protected from apoptosis induced by chemotherapeutic agents compared with those grown on plastic (Figure 3). Apoptosis was assessed by several methods, including morphology, Acridine Orange/ethidium bromide staining and immunoassay of cytoplasmic histone-associated DNA. Non-specific adhesion of SCLC cells to poly(l-lysine) did not protect cells from chemotherapy-induced apoptosis. Furthermore, co-incubation with a function-blocking β1 integrin antibody, but not with isotype-matched control antibodies, abolished the ECM-mediated protection. Taken together, these data showed that ECM-mediated protection from chemotherapy-induced apoptosis was integrin-dependent.

MECHANISM OF ECM-MEDIATED PROTECTION OF SCLC CELLS FROM CHEMOTHERAPY-INDUCED APOPTOSIS

Having made the observation that ECM proteins protect SCLC cells from chemotherapy-induced apoptosis, we attempted to dissect the molecular mechanism underlying this phenomenon. Our group has demonstrated previously that protein tyrosine kinase (PTK) activity regulates SCLC cell survival [16]. We found that adhesion of SCLC cells to fibronectin stimulated PTK activity in both untreated and etoposide-treated cells. This effect could be completely blocked by pretreatment either with tyrphostin-25 (a selective PTK inhibitor) or with a function-blocking β1 antibody.

Figure 4 Effect of fibronectin on etoposide-induced caspase-3 activation

H69 SCLC cells were seeded at a density of 5 × 10⁴ cells/ml into 24-well plates in the presence or absence of pre-coated fibronectin (Fn; 20 µg/ml) with or without etoposide (25 µg/ml), tyrphostin-25 (25 µM), benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD; 100 µM) or sodium orthovanadate (Na₃VO₄; 200 µM). After 24 h, caspase-3 activity was determined using a commercially available kit. AU, arbitrary units.

The final common pathway for the induction of apoptosis centres on caspase-3, which is formed from the proteolytic cleavage of pro-caspase-3. Following activation, caspase-3 cleaves the chaperone inhibitor of caspase-activated deoxyribonuclease, releasing DNase activity, and it is this that causes DNA fragmentation. While etoposide stimulated caspase-3 activation and apoptosis in cells grown on plastic, both processes were completely blocked either by a tyrosine phosphatase
inhibitor or by the caspase inhibitor benzoyl oxy carbonyl-Val-Ala-D,L-Asp-fluoromethylketone (‘Z-VAD’) (Figure 4). However, when SCLC cells were grown on fibronectin, etoposide-induced caspase-3 activation was markedly reduced (Figure 4). This ECM-mediated protection could be inhibited by a function-blocking β1 antibody and by tyrphostin-25. Taken together, these data showed that β1-integrin-dependent resistance to chemotherapy was mediated by a PTK-dependent mechanism.

Finally, we assessed whether adhesion to ECM proteins had any effect on topoisomerase II activity. Etoposide initiates its cytotoxic action by acting as a specific inhibitor of DNA topoisomerase II. Topoisomerase II is a nuclear enzyme that effects unknotting and relaxation of supercoiled DNA molecules by a process of introducing transient double strand breaks through which the strands of an intact helix can pass. Topoisomerase poisoning results in the trapping of enzyme molecules on DNA as cleavable complexes and the generation of potentially lethal lesions. We found that neither adhesion to fibronectin nor treatment with tyrphostin-25 affected the activity of topoisomerase II in basal or etoposide-treated cells. These data suggest that ECM-induced protection from chemotherapy-induced apoptosis acts at a point downstream of DNA damage.

In summary, these data show that, in vivo, SCLC cells are surrounded by an extensive stroma of ECM proteins at both primary and metastatic sites. SCLC cells adhere to ECM proteins in a predominantly β1-integrin-mediated fashion. When SCLC cells are adhered to ECM proteins, they are protected from the pro-apoptotic effects of chemotherapeutic agents. The mechanism underlying this phenomenon is due to an ECM-induced increase in tyrosine phosphorylation, which blocks chemotherapy-induced caspase activation.

DISCUSSION

The extent of ECM deposition around SCLC cells in vivo has not been fully appreciated before. Unlike laminin and collagen IV expression, which was confined to basement membranes, tenascin and fibronectin were expressed in areas of reactive host tissue scarring and within broad stromal bands around groups of tumour cells. Tenascin is transiently expressed in many developing organs, but is gerally absent from normal adult tissues. However, tenascin is re-expressed in adult tissues that are actively remodelling following denervation [17] and in wound healing [18], as well as in the stroma of a wide variety of tumours, such as gliomas [19], breast carcinomas [20] and basal cell carcinomas [21]. Similarly, neo-expression of some fibronectin isoforms, while absent from normal interstitium, occurs in tumour stroma [22]. The neo-expression of tenasin and fibronectin in the interstitium of SCLC tumours suggests that the tumour cells may condition their stroma in order to support their own growth. SCLC cells produce a variety of growth factors, cytokines and inflammatory mediators, including insulin-like growth factor-1, gastrin-releasing peptide and interleukin-8, which may, through complex autocrine and paracrine interactions, be able to modulate the immediate environment of SCLC cells [23,24]. Insulin-like growth factor-1 and gastrin-releasing peptide are mitogenic for both SCLC cells and fibroblasts. Interleukin-8 is an essential angiogenic factor for non-small-cell lung cancer in nude mice [25]. Therefore, in vivo, SCLC may create a specialized environment as a consequence of autocrine and paracrine effects that, using an analogy with inflammation, likens SCLC to ‘a wounding reaction’, with the laying down and remodelling of ECM, growth factor release and neovascularization.

We have found that ECM proteins are able to protect SCLC cells from chemotherapy-induced apoptosis. There has been one other report of laminin-induced chemoresistance of SCLC cells [26]. We have now extended these findings to show that this is a general property of several ECM proteins found in increased amounts in SCLC tumours in vivo. The finding that ECM-mediated chemoresistance could be abrogated by a function-blocking antibody against β1 integrin showed that the effect is β1-integrin-mediated. Another group has reported that etoposide-induced DNA strand breakage in xenograft tumour-derived endothelial cells could be inhibited by culturing these cells on gelatin, collagen IV, laminin, fibronectin or the integrin ligand hexapeptide Gly-Arg-Gly-Asp-Ser-Pro, but not on the integrin peptide Gly-Arg-Ala-Asp-Ser-Pro [27]. DNA damage was also inhibited when tumour-derived endothelial cells were plated on surfaces coated with antibodies to α5, β1 and β3 integrin subunits, and by clustering integrins with soluble antibodies. Similarly, Dalton’s group have reported that fibronectin-mediated adhesion of myeloma cells confers a survival advantage following exposure to cytotoxic drugs [28,29]. Again, α4/β1- and α5/β1-integrin-mediated adhesion inhibited drug-induced apoptosis.

In our system, adhesion of SCLC cells to ECM proteins induced tyrosine phosphorylation, and inhibition of tyrosine kinase activity abrogated ECM-induced chemoresistance and promoted apoptosis. Furthermore, etoposide-induced apoptosis could be blocked by the presence of the tyrosine phosphatase inhibitor sodium orthovanadate. Protein tyrosine phosphorylation has been described previously in response to cell attachment to ECM proteins [30] and on integrin clustering [31,32]. PTK activity has been reported to regulate apoptosis: Abl protein, a non-receptor-type tyrosine kinase, inhibited cytokine-withdrawal-mediated [33] or Fas-mediated [34] apoptosis. Furthermore, inhibition of
PTK activity by inhibitors, such as erbstatin, genistein or tyrphostin-25, has been shown to induce apoptosis in SCLC cells [35,36].

With our developing understanding of mechanisms that regulate apoptosis, it is becoming increasingly clear that chemotherapeutic agents act through similar mechanisms. Kaufmann et al. [37] identified proteolytic cleavage of poly(ADP-ribose) polymerase in response to etoposide. It now appears that many inducers of cell death, including cytokines and chemotherapeutic agents, ultimately converge on the activation of this and related proteases, which then trigger the terminal and execution stages of apoptosis. Our work shows that etoposide-induced caspase-3 activation is modulated by ECM-induced tyrosine phosphorylation. ECM-mediated protection from etoposide-induced caspase-3 activation could be blocked either by a β1 integrin function-blocking antibody or by a tyrosine kinase inhibitor. Interestingly, Boudreau et al. [38] reported that apoptosis of CID-9 mammary epithelial cells could be induced by antibodies to β1 integrins or by overexpression of stromelysin-1, which degrades the ECM. Expression of interleukin-1β-converting enzyme (ICE) was correlated with the loss of ECM, and inhibitors of ICE activity prevented apoptosis. These results suggested that ECM regulates apoptosis in mammary epithelial cells through an integrin-dependent negative regulation of ICE expression. Similarly, Simizu et al. [39] showed that induction of apoptosis by erbstatin in SCLC cells resulted in the activation of caspase-3(-like) proteases. They too concluded that, since erbstatin and herbimycin A do not inhibit other protein kinases, tyrosine kinase activity or tyrosine-phosphorylated proteins may be a negative regulator of caspase activity. The exact mechanism by which this occurs is not known. It would seem likely that there may be one or more tyrosine-phosphorylated proteins involved. One possible candidate is phosphoinositide 3-kinase, a phospholipid that requires tyrosine phosphorylation for full activation. There have been a number of publications linking the phosphoinositide 3-kinase/protein kinase B pathway to cell survival and protection from apoptosis [40–42]. One might speculate that the final mediator inhibiting caspase-3 activation is one of the Bcl-2 family of proteins. Protein kinase B phosphorylates Bad, thereby preventing it from inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-XL. It has been demonstrated that Bcl-XL can inhibit caspase-3 activation [43]. Although our group has not been able to demonstrate any alteration in Bcl-2 expression in SCLC cells adhered to fibronectin, Simizu et al. [39] demonstrated that Bax mediated erbstatin-induced caspase-3 activation. Bax, another member of the Bcl-2 family, promotes apoptosis and antagonizes the function of Bcl-2.

Based upon our work and the current literature, the following model for the development of chemoresistance in SCLC cells is proposed (Figure 5). Etoposide acts by inhibiting the re-annealing action of topoisomerase II, causing multiple DNA nicks. This persistent DNA damage results in cell cycle delay in S phase and G2/M, with subsequent caspase-3 activation leading to apoptosis. However, β1-integrin-mediated cell adhesion to ECM proteins results in tyrosine phosphorylation, which, despite persistent chemotherapy-induced DNA damage, prevents caspase activation and apoptosis. In this way, tumour cells bound to ECM escape chemotherapy-induced cell death; then, with subsequent genetic damage, drug-resistant clones may be selected. We believe that such a mechanism may be responsible for the initial resistance of SCLC cells to chemotherapy. Resistance to chemotherapy conferred by P-glycoprotein and MRP may develop later in the evolution of chemoresistance, although overexpression of MRP is not common in SCLC [44].

The exact point at which the ECM-mediated tyrosine phosphorylation pathway impinges upon the chemotherapy-induced pro-apoptotic pathway is unclear at present. Increased tyrosine phosphorylation had no effect on etoposide-induced topoisomerase II inhibition, indicating that it acts downstream of DNA damage. One possible mechanism is that ECM-induced tyrosine phosphorylation prevents cell cycle arrest by modulating cyclin-dependent kinase activity. Progression from G2 into M phase is controlled in part by a cyclin-dependent kinase (cyclin B/CdkI) that is regulated by tyrosine phosphorylation [45].

In conclusion, we believe that our model may begin to explain the partial responses and local recurrence of SCLC that often occurs after chemotherapy. A number of specific inhibitors of tyrosine kinases have been
described [46]. If we can define the exact pathways mediated by the ECM, it may be possible to selectively target key signalling molecules and block the ‘protective pathway’, thereby augmenting the effects of conventional chemotherapeutic drugs.

REFERENCES


R. C. Rintoul and T. Sethi

424