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Chemotherapy often relies on cancer cell death resulting from DNA-damage. The p53 tumor suppressor pathway that is an important player in DNA-damage response is frequently inactivated in cancer. Genotoxicants also activate DNA-damage-independent stress pathways and activity of oncogenic signaling and adhesive interactions with the cancer microenvironment can have a strong impact on chemosensitivity. Here, we have investigated how two different oncogenes modulate the response to genotoxicants in the context of two classes of integrin adhesion receptors. Epithelial cells expressing either β1 or β3 integrins, in which p53 activity is suppressed, undergo G2 arrest but show very little apoptosis following treatment with cisplatin and several other genotoxicants. The apoptotic response is strongly enhanced by the c-Src[Y530F] oncogene in cells expressing β1 integrins. However, when these cells are engineered to express β3 integrins instead, no such sensitization occurs. The H-Ras[G12V] oncogene fails to sensitize, regardless of the integrin expression profile. The enhanced sensitivity induced by c-Src[Y530F] in the context of β1 integrins is p53-independent, but instead, occurs through increased endoplasmic-reticulum-stress and caspase-3 activation. Our data implicate that the expression profiles of oncogenes and integrins strongly affect the response to chemotherapeutics and may thus determine the efficacy of chemotherapy.

INTRODUCTION

Most chemotherapeutic treatments rely on cancer cell death in response to DNA-damage, although many genotoxic compounds also activate stress pathways independent of DNA-damage, for instance by inducing reactive oxygen species or binding to proteins within cells. Normally, the induction of DNA-damage by genotoxic agents triggers apoptosis initiated by DNA checkpoint proteins such as ATM, ATR and p53 (Roos and Kaina, 2006). However, the vast majority of cancers harbor (epi)genetic changes that inactivate the Rb and p53 tumor suppressor pathways. Loss of p53 may suppress the apoptotic response to DNA-damage and cause resistance to therapy. Conversely, accelerated cell cycle progression and compromised repair in the absence of Rb and p53 may lead to accumulation of DNA damage, causing sensitization to therapy. The final outcome of treatment with genotoxicants will also be determined to a large extent by other oncogenic pathways present in the tumor cells as well as by interactions with the cancer microenvironment.

The cancer microenvironment, including the extracellular matrix (ECM), is known to critically modulate the apoptotic response to treatment with genotoxic compounds (Bissell and Radisky, 2001; Morin, 2003). For instance, ovarian cancer cells were shown to be able to remodel the ECM thereby favoring survival in the presence of the genotoxic compound...
cisplatin (Sherman-Baust et al., 2003). Also, increased deposition of the ECM proteins fibronectin, collagen IV, and laminin was shown to confer resistance of small cell lung cancer to chemotherapeutic agents (Sethi et al., 1999). (Cancer) cells interact with the ECM through a family of receptors called integrins. These heterodimeric transmembrane receptors couple the ECM microenvironment to the cytoskeleton and are able to recruit multiple adaptor and signaling proteins to sites of adhesion (Hynes, 2002). Besides their essential role in cell adhesion, integrins are important for providing survival and proliferative signals through extensive cross-talk with growth factor receptors (Yamada and Even-Ram, 2002).

Changes in integrin expression levels are frequently associated with tumor growth and progression (Mizejewski, 1999), and it has been suggested that the tumor microenvironment directly modulates integrin signaling thereby promoting malignancy (Paszek et al., 2005). Interestingly, changes in expression levels of certain integrins have also been reported to either promote or reduce sensitivity to genotoxic compounds (Cordes, 2006). Indeed, drugs targeting integrin function are considered promising adjuvants to sensitize tumor cells to chemotherapeutics (Damiano, 2002; Huveneers et al., 2007a). However, it remains largely unclear how such perturbations modulate drug sensitivity and how the pattern of mutations in tumor suppressors and oncogenes modulates these effects.

To address these issues, we made use of a panel of cell lines derived from poorly adhesive, integrin β1 KO cells that were immortalized using SV40 Large T (inactivation of p53 and Rb pathways). In the absence or presence of oncogenes (activated H-Ras or c-Src) we restored adhesion by either re-expressing β1 or by enhancing β3 expression to compensate for the absence of β1. When this panel of cell lines was subjected to treatment with a variety of DNA-damaging and non-DNA-damaging cytotoxicants, the apoptotic response to these compounds revealed a remarkably strong dependency on both the oncogene and the integrin profile. Our data indicate that activated c-Src but not H-Ras sensitizes cells to p53-independent, caspase 3-dependent, endoplasmic reticulum (ER) stress-related apoptosis only in the context of β1 integrins. These results delineate a novel oncogene- and integrin-controlled signaling pathway that determines (cancer) cell sensitivity to chemotherapeutic agents.

**MATERIAL AND METHODS**

**Cell lines, plasmids, antibodies and compounds**

The β1-deficient GE11 cells were previously described (Gimond et al., 1999). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. LZRS bicistronic retroviral expression plasmids encoding SrcYF, RasGV, human integrin β1 and β3 were described before (Danen et al., 2002; Huveneers et al., 2007b). To ensure identical expression of SrcYF (c-Src[Y530F]) or RasGV (H-RasG12V) in cell lines, we first generated GESrcYF and GERasGV single cell clones and subsequently expressed β1 or β3 integrin subunits using retroviral transduction and bulk sorting by FACS. The following antibodies were used: mouse monoclonal anti-p53 (Pab240, Santa Cruz Biotechnology) and anti-α-tubulin (DM1A, Sigma); rabbit polyclonal anti-p21 (C-19, Santa Cruz Biotechnology), anti-phospho-p53 (Ser15, Cell Signaling), anti-caspase-3 (Cell Signaling), and anti-phospho-eIF2α (Ser51, Cell Signaling). Cisplatin, etoposide, menadione, mitomycin C, and thapsigargin were all purchased from Sigma. Salubrinal came from Calbiochem, vincristine from TEVA Pharmachemie, and z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk) from Bachem. S-(1,2-dichlorovinyl)-L-cysteine (DCVC) was synthesized as previously described (Hayden and Stevens, 1990). Compounds were diluted in complete medium and supplied to the cells as indicated in the text.

**siRNA transfections**

Cells were plated at approximately 40% confluency and were transfected the following day using DharmaFECT 1 reagent and a final concentration of 50 nM of p53 SMARTpool siRNA (M-040642-00) or siCONTROL non-targeting siRNA#2 (D-001210-02) purchased from Dharmacon. 24 hours post-
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Figure 1. β1 integrins sensitize cells to DNA-damaging compounds in presence of the Src oncogene. (A) Graphs indicate the percentage of GE11 cell types in G1/G0, S or G2/M phase of the cell cycle after treatment with DNA-damaging compounds. Ectopic expression of integrin β1, integrin β3, c-Src[Y530F] or Ras[G12V] is indicated. Treatments were performed during 8 hours in complete culture medium supplied with cisplatin (5 μM), mitomycin C (1 μM) or etoposide (10 μM). Control indicates treatment with DMSO. Cell cycle was determined by flow cytometry analysis of DNA content. (B) Graphs indicate the percentage of apoptosis of GE11 cell types induced by DNA-damaging compounds. Treatments were performed during 8, 16 or 24 hours in complete culture medium supplied with DNA-damaging compounds used at different concentrations: cisplatin (5, 10, 20 μM; indicated by +, ++, +++ respectively), mitomycin C (1, 5, 10 μg/ml), or etoposide (10, 20, 40 μM). Apoptosis was determined by flow cytometry analysis of DNA content.
transfection, cells were replated and used for subsequent experiments.

**Cell cycle analysis and apoptosis assay**
Floating and trypsinized adherent cells were pooled and fixed in 80% ethanol overnight at -20°C. Cell pellets were subsequently washed with PBS-EDTA (1 mM) twice, resuspended in PBS-EDTA containing 7.5 μM propidium iodide and 40 μg/ml RNase A. After 45-min incubation at room temperature, the cell cycle was analyzed by flow cytometry on a FACS Calibur (BD Biosciences). Cell cycle analysis and the percentage of apoptotic cells were determined using CellQuest software (BD Biosciences).

**Western blot analysis**
For Westerns total cell lysates were prepared in ice cold TSE (10 mM Tris-HCL, 250 mM Sucrose, 1 mM EGTA pH 7.4 and protease inhibitors), sonicated, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked in 5% BSA TBS-T, incubated with primary and secondary antibodies, followed by ECL or ECLplus reaction (GE Healthcare). ECL signal was detected using films or a Typhoon 9400 (Molecular Dynamics).

**RESULTS**
β1 integrin-deficient GE11 cells were used to investigate the role of integrins in the cellular response to genotoxic agents. These cells adhere weakly and we used retroviral transduction to restore adhesion by re-expressing β1 (GEβ1) or compensate for the absence of β1 by enhancing β3 expression (GEβ3) (Danen et al., 2002). Exposure of GEβ1 and GEβ3 cells to the DNA-damaging agents cisplatin, mitomycin C, etoposide, or doxorubicin caused a cell cycle arrest at the G2 phase in both β1 and β3 expressing cell types (Figure 1A top two panels and data not shown). Induction of apoptosis was minimal after treatment with these genotoxic agents, which can be explained by Large T-mediated inactivation of the p53 tumor suppressor pathway, mimicking the situation in cancer cells (Figure 1B top two panels). We next generated the following cell types by retroviral transduction: GESrcβ1 and GESrcβ3 cells, that express a constitutively primed form of the proto-oncogene c-Src[Y530F] together with β1 or β3 integrins; and GERasβ1 and GERasβ3 cells that express oncogenic H-Ras[G12V] in the context of these integrin classes (Huveneers et al., 2007b). Regardless of the integrin expression profile, genotoxic treatment of these oncogene-transformed cells led to a similar G2 cell cycle arrest as observed in the absence of oncogenes (Figure 1A). However, a remarkable strong induction of apoptosis was observed specifically in GESrcβ1 cells (Figure 1B). By contrast, Src did not promote apoptosis in β3 expressing cells. Importantly, these differences could not be explained by different levels of Src expression; the GESrcβ variant was made first, followed by expression and bulk FACS sorting for the integrin subunits, ensuring identical expression levels of Src in the GESrcβ1 and GESrcββ3 lines (Huveneers et al., 2007b). Moreover, this effect was specific for Srcβ: transformation by Ras[GV] only weakly affected the response to DNA-damaging agents, regardless of the integrin expression profile (Figure 1). Taken together, these results indicate that expression of an oncogenic c-Src mutant, but not of H-Ras, leads to a strong sensitization to genotoxic compounds in the context of β1, but not β3 integrins.

For further studies we focused on cisplatin as a marker compound that induces prominent apoptosis in GESrcβ1 cells, but not in other oncogene/integrin combinations (Figure 2A). The amount of apoptotic GESrcβ1 cells after cisplatin exposure was concentration dependent, reaching a maximum level of cell death at a concentration of 25 μM, whereas no evident increase of apoptosis was observed in the other cell types (Figure 2B,C). We investigated if the enhanced apoptosis in the presence of Srcβ in combination with β1 integrins occurred at the level of caspase-3 activation. Indeed, cisplatin readily induced caspase-3 cleavage in GESrcβ1 cells whereas caspase-3 cleavage in cells lacking Srcβ or in cells expressing Srcβ in the context of β3 was much weaker (Figure 3A). In line with the activation of caspase-3 in cisplatin-induced apoptosis, a pan-caspase inhibitor, z-VAD-fmk strongly reduced cisplatin-induced apoptosis of GESrcβ1.
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Figure 2. Cisplatin induces apoptosis of SrcYF-transformed cells expressing β1 integrins. (A) Analysis of DNA content by flow cytometry demonstrating the amount of GE11 cells expressing indicated constructs in apoptosis and in different phases of the cell cycle after treatment with 10 μM cisplatin for 24 hours. Control indicates treatment with DMSO. (B) Dose-response curves indicating the increase in percentage of apoptosis, induced by treatment with various concentrations of cisplatin for 24 hours. (C) Graphs indicate the average percentage of apoptotic cells ± standard deviation (SD) of three independent experiments (n=3) induced by treatment with 25 μM cisplatin for 24 hours.

cells (Figure 3B). These results indicate that the increased sensitivity of GESrcYFβ1 cells to cisplatin occurs through a caspase-mediated apoptosis program.

DNA-damage caused by chemotherapeutics such as cisplatin induces an apoptotic response, which can be mediated through several signaling pathways. Of these, signaling to the transcription factor p53 is one of the major pathways mediating DNA-damage induced apoptosis (Jordan and Carmo-Fonseca, 2000; Roos and Kaina, 2006). Large T expression in GE11 cells mimics inactivation of the p53 pathway seen in the majority of cancers, but we examined if there was a potential residual role for p53 in cisplatin-induced apoptosis of GESrcYFβ1 cells. Irrespective of the integrin/oncogene expression status, cisplatin treatment induced phosphorylation of p53 on Ser15 and induced an increase in the expression of the p53 target gene, p21 thus revealing a fraction of p53 that is not sequestered by Large T (Figure 4A). To directly test if p53-mediated effects were involved in cisplatin-induced apoptosis of GESrcYFβ1 cells, we silenced p53 gene expression. Introduction of si-p53 only weakly reduced total p53 protein expression after 72 hours (presumably due to the high stability of the major fraction of Large T-sequestered, inactive p53), but it abolished the induction of phosphorylated (active and presumably less stable) p53 and its target, p21 upon cisplatin treatment (Figure 4B). However, despite this elimination of the p53 response, si-p53 failed to inhibit cisplatin-induced apoptosis in GESrcYFβ1 cells (Figure 4C). These results indicate that SrcYF sensitizes cells to cisplatin in the context of β1 integrins, through a p53-independent pathway.
Figure 3. Increased caspase activity mediates cisplatin-induced apoptosis of GESrcYFβ1 cells. (A) Western blot analysis of caspase-3 cleavage in lysates of GE11 cells expressing indicated constructs treated with 10 μM cisplatin for indicated exposure times. (B) Graphs indicate the average percentage of apoptosis ± SD of two independent experiments performed in duplo after 10 μM cisplatin treatment (24 hours exposure) of GESrcYFβ1 cells with or without z-VAD-fmk caspase inhibitor preincubation.

Figure 4. β1 integrins sensitize SrcYF-transformed cells to cisplatin independently of p53 signaling. (A) Western blot analysis of total p53, p-p53 (phospho Ser15), p21 and tubulin (loading control), in lysates from GE11 cells expressing indicated constructs with or without 25 μM cisplatin treatment (24 hours exposure). (B) Western blot analysis of lysates from GESrcYFβ1 cells treated with 25 μM cisplatin (24 hours exposure) transfected with p53 or control siRNAs. (C) Graphs indicate the average percentage of apoptosis induced by cisplatin (25 μM; 24 hours exposure) in GESrcYFβ1 cells transfected with p53 or control siRNAs ± SD of three independent experiments (n=3).
Besides causing DNA-damage, genotoxic agents can induce other cytotoxic stresses such as oxidative-, mitochondrial- or ER-stress that may contribute to the clearance of tumor cells (Asakura and Ohkawa, 2004; Chen et al., 2007; Linder and Shoshan, 2005). Since we ruled out a role for the classical p53-mediated DNA-damage response pathway, we wondered if activation of any of the DNA-damage-independent pathways could underlie the enhanced sensitivity to cisplatin upon SrcYF expression in the context of β1 integrins. We first tested if the response to a variety of different cytotoxic agents mimicked the pattern of response to cisplatin in our panel of cell lines. Oxidative-stress inducers (H₂O₂ and menadione) readily induced cell death as judged by cell rounding or detachment, but this response was not related to integrin expression profiles or SrcYF expression (Figure 5A). Vincristine, a disruptor of the microtubule network only weakly affected viability of these cells and DCVC, a nephrotoxicant inducing oxidative- and mitochondrial-stress (van de Water B. et al., 1999), caused cell death in β1-expressing cells, irrespective of the expression of SrcYF.

On the other hand, the response to treatment with thapsigargin, a specific inducer of ER-stress (Linder and Shoshan, 2005), mimicked the response to cisplatin: thapsigargin readily triggered cell death of GE SrcYF/β1 cells while it did not or only weakly affected GEβ1 or GE SrcYF/β3 cell survival (Figure 5A). FACS analysis of DNA content confirmed this effect and indicated that thapsigargin, like cisplatin, selectively induced apoptosis of GE SrcYF/β1 cells (Figure 5B). These findings suggested that increased ER-stress-related pathways could underlie the enhanced sensitivity to cisplatin upon SrcYF expression in the context of β1 integrins.

To substantiate the involvement of ER-stress, we made use of a phosphatase inhibitor, salubrinal that has been shown to specifically prevent ER-stress-induced apoptosis at the level of eIF2α (Boyce et al., 2005). As expected, thapsigargin-induced apoptosis was reduced to a level that was close to that observed in the much less-sensitive GE SrcYF/β3 cells (Figure 5B). We next tested if enhanced ER-stress could underlie cisplatin-induced apoptosis in GE SrcYF/β1 cells. Indeed cisplatin-treatment led to an increase in phosphorylation of eIF2α, pointing to an induction of ER-stress (Figure 5C). Moreover, in agreement with the notion that ER-stress was involved in the increased sensitivity of GE SrcYF/β1 cells to cisplatin, salubrinal readily suppressed apoptosis of GE SrcYF/β1 cells treated with cisplatin (Figure 5D). Altogether, these findings demonstrate that depending on the integrin expression profile, oncogenic c-Src but not H-Ras can sensitize cells to cisplatin-induced ER-stress-mediated apoptosis.

**DISCUSSION**

The development and application of specific integrin-blocking strategies as treatment against cancer is promising. Interactions with the extracellular matrix have been described to provide survival and proliferation signals and integrins can mediate resistance of tumor cells to genotoxic injury caused by chemotherapeutic agents, a process referred to as cell adhesion-mediated drug resistance (Damiano et al., 1999). Little is known about roles for distinct integrins in these processes or how the genetic make-up of the tumor influences the impact that integrins can have. Previous experiments using β1-deficient cells, indicate that in the absence of oncogenic mutations, the loss of β1 integrins sensitizes to radiation-induced genotoxic injury (Cordes et al., 2006). In our current study we have compensated for the weak adhesion in the absence of β1 by enhancing the expression of β3, and we rule out a specific requirement for β1 integrins in protection against genotoxicants. We have investigated how two different classes of integrins affect chemosensitivity in the absence or presence of two different oncogenes. Our results reveal extensive cross-talk between the integrin expression profile and active oncogenic pathways in determining chemosensitivity. Moreover, we find that it is not the classical DNA-damage response that is controlled by these parameters but rather, the ability of genotoxic...
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Figure 5. Cisplatin induces apoptosis of GESrc<sup>YF</sup>β1 cells through an elevated ER-stress response. (A) Table summarizes the approximate amount of cell death (judged by cell rounding and detachment) induced by various cytotoxic compounds. GE11 cells expressing indicated constructs were treated with 100 μM H<sub>2</sub>O<sub>2</sub> (oxidative stress inducer), 250 μM S-(1,2-dichlorovinyl)-L-cysteine (DCVC; nephrotoxicant inducing oxidative- and mitochondrial-stress), 100 μM menadione (oxidative stress inducer), 20 nM vincristine (disrupts microtubules) or 5 μM thapsigargin (ER-stress inducer). (B) Graphs indicate the percentage of apoptosis (n=3) of GE11 cells expressing indicated constructs induced by treatment of the ER-stress inducer thapsigargin at indicated concentrations (24 hours exposure). Salubrinal was used to inhibit ER-stress induced in GESrc<sup>YF</sup>β1 cells. (C) Western blot analysis of phosphorylated eIF2α and tubulin in lysates of cisplatin treated GESrc<sup>YF</sup>β1 cells (8 hours exposure). (D) Graphs indicate the average percentage of apoptosis ± SD of two independent experiments performed in duplo after cisplatin treatment of GESrc<sup>YF</sup>β1 cells in the presence or absence of the ER-stress inhibitor salubrinal.
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compounds to induce ER-stress-mediated apoptosis.

Our findings indicate that the increased levels of Src activity that are often seen in various types of cancer may enhance their chemosensitivity. However, the role of Src in regulating the balance between survival and apoptosis in cancer cells is complex. Cisplatin-induced elevation of the activities of epidermal growth factor receptor (EGFR) and c-Src in cancer cells has been reported to act as a survival response, and inhibition of these activities increased cisplatin-induced cell death (Benhar et al., 2002; Pengetnze et al., 2003). On the other hand, cisplatin-induced apoptosis has been shown to depend on EGFR and c-Src activity in other systems and expression of v-Src can induce sensitivity to cisplatin analogues, pointing to a pro-apoptotic and drug-sensitizing effect of enhanced Src activity (Arany et al., 2004; Turkson et al., 2004; Webb et al., 2000). Our current findings indicate that integrin-mediated interactions with the microenvironment can determine how Src affects chemosensitivity.

Rather than the classical DNA-damage response, we identify Src as a regulator of the ER-stress response that can also be induced by genotoxic compounds. It has been previously observed that cisplatin can induce apoptosis by stimulating ER-stress (Mandic et al., 2003). Moreover, Src activation has also been implicated in p53-independent pathways, including those leading to apoptosis (Ulianich et al., 2008; Webb et al., 2000). Our results extend these findings and indicate that Src activity can enhance cisplatin-induced apoptosis under conditions where p53-dependent pathways are suppressed by inducing ER-stress.

Given the strong attention that αvβ3 currently receives as a potential target for cancer therapy in combination with chemoradiotherapy (Huveneers et al., 2007a), it is intriguing that enhanced Src activity indeed fails to sensitize cells to cisplatin in the context of αvβ3. Previously, we have shown that Src-mediated tumor growth is strongly supported by an interaction with αvβ3 (Huveneers et al., 2007b). Our current study extends these findings and demonstrates that survival/apoptosis signaling by Src in the presence of chemotherapeutic compounds is similarly subject to tight regulation by the integrin expression profile. To some extent, differential regulation of Src-induced cytoskeletal organization and effects on adhesion/spreading may underlie the strong effect of the integrin expression profile: high levels of αvβ3 protect against the inhibition of cell spreading that is a hallmark of Src-transformed cells (Huveneers et al., 2008). Nevertheless, no differences in proliferation are observed between GESrc YF/αvβ3 cells and any of the other cell lines tested in the absence of drugs, indicating that potential effects through adhesion signaling only become evident upon drug-exposure.

Altogether, our findings indicate that cross-talk between integrins and oncogenes can have dramatic effects on chemosensitivity of cancer cells. Expanding studies along these lines could provide the information that will enable us to predict for which tumors integrin antagonists may be successfully used in combination with classical chemotherapy to stop cancer growth and progression.

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