A CD44 Survival Pathway Triggers Chemoresistance via Lyn Kinase and Phosphoinositide 3-Kinase/Akt in Colon Carcinoma Cells

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ABSTRACT

A major obstacle to successful treatment of colorectal cancer is chemotherapy resistance. Enhanced expression of variant CD44 isoforms has been associated with aggressive tumor behavior, prompting the question of whether signaling from this receptor might modulate drug sensitivity. Activation of variant CD44 in colon carcinoma cell lines triggered resistance to the drug 1,3-bis(2-chloroethyl)-1-nitrosurea. Resistance was induced by monoclonal antibodies directed against epitopes independent of the hyalurionate-binding region but was not triggered by identical treatment of a carcinoma line expressing the standard CD44 isoform. We observed that variant CD44 produced activation of the src-family tyrosine kinase lyn. Moreover, overexpression of dominant-active lyn recapitulated chemoresistance via a pathway shown to involve activation of phosphoinositide 3-kinase and Akt. These results establish a novel role for CD44 in determining survival of colon carcinoma cells through lyn kinase and Akt. The ability to suppress apoptosis might play a critical role in the onset and development of colorectal malignancies.

INTRODUCTION

CD44, the principle HA receptor in a variety of cell types (1, 2), plays a role in a diverse range of physiological and pathological processes, including cell-cell and cell-extracellular matrix interactions, cell migration, lymphocyte homing, leukocyte activation, hemopoesis, presentation of chemokines and growth factors, and metastatic spread (reviewed in Refs. 3–6). The complexity of CD44 function has been linked to its structure, although functional characterization of the different isoforms which constitute this family is still limited. The CD44 molecule is known to display extensive size heterogeneity, ranging from the standard 85–95 kDa form (CD44s) to larger variant isoforms (CD44v) of 200 kDa or more. These size differences can be partially attributed to posttranslational modifications, as all isoforms of CD44 are highly glycosylated (2). However, most variants are generated by alternative splicing of at least 12 exons, leading to changes in the core protein (7). In addition to functioning as a HA receptor, CD44 can function as a receptor for other ligands, including collagen (8) and fibronectin (9). By analogy with other families of adhesion receptors able to bind multiple ligands, e.g., the integrins (10), this raises the possibility that individual CD44 isoforms can transduce multiple signals and activate different pathways in response to binding discrete ligands. Further, because of the structural diversity of CD44, it is likely that the multiple domains of this protein can influence the signal-transducing function of the molecule as a whole.

Enhanced expression of variant CD44 isoforms is seen in tumors of the colon, as compared with corresponding normal tissue (11, 12). In normal colonic epithelium, the standard CD44s isoform is expressed by the dividing cells at the base of the colonic crypt but is absent from the nonproliferating epithelium of the upper crypt and luminal surface (13). In contrast to this restricted expression, invasive carcinomas and metastases express variant CD44 proteins, including the CD44v6 isoform which has been shown to confer metastatic behavior on rat pancreatic cells (14). Interestingly, metastasis-related variants have also been found at relatively early stages in colorectal carcinogenesis, on adenomatous polyps, and adenomas (12, 13), suggesting the existence of selective advantages linked to variant CD44 expression. Moreover, this receptor is associated with poor prognosis in human colon cancer (15). The biological basis of these effects of variant CD44 expression is not known.

In addition to the functions outlined above, a fundamental role in promoting cell survival is emerging for CD44. It has been shown that one of the early features of CD95 (Fas/Apo-1)-induced apoptosis in colon carcinoma cell lines was the shedding of CD44 from the cell surface (16). It was proposed that this loss of CD44 was likely to be an important factor in the death program, not only because of its role in cell adhesion but to a direct contribution of survival-sustaining signals (16). Further, CD44 has been reported to have an antiapoptotic effect in lymphocytes, where it protected against anti-CD3 and dexamethasone-induced, but not radiation-induced, cell death (17). The actual mechanism of protection was not determined, but it appeared not to be related to overexpression of bcl-2. In a previous study, we found that activation through a variant form of CD44 (CD44v6) could suppress apoptosis, induced by an anti-integrin antibody (18). Using the LIM 1863 colon carcinoma cell line, which usually grows as structured organoids around a central lumen, we demonstrated that ligation of CD44 by immobilized antibody could induce a monolayer morphology in these cells, and this was accompanied by an acquired resistance to apoptosis.

The serine/threonine kinase Akt is now a well-recognized mediator of cell survival in response to a number of stimuli, particularly growth factor signaling and cellular adhesion (19–22). The PI3K3-dependent activation of Akt has been established as a critical survival pathway through a number of studies involving overexpression of Akt, functional interference with dominant-negative constructs, and pharmacological inhibitors of PI3K (19, 20, 23). Several mechanisms have recently been described for how Akt suppresses apoptosis. One involves the phosphorylation of the proapoptotic protein BAD, which in turn is scavenged by the cytosolic protein 14-3-3 and neutralized (24). Another is dependent on the phosphorylation of caspase-9, one of the “executioner” caspases in the apoptotic cascade, thereby suppressing its function (25). Another substrate of Akt, glycogen synthase kinase-3, has also been identified as a key target of PI3K signaling leading to the prevention of apoptosis (26). More recently, another model has been proposed, whereby Akt has been shown to phosphorylate and suppress the activity of FKHR1, a member of the Fork-
head family of transcription factors, the dephosphorylated form of which is capable of translocating and activating proapoptotic proteins (27). These proposed actions of Akt are not mutually exclusive, and it is likely that additional mechanisms will emerge as this signaling pathway becomes better understood.

In the present study, we have examined the biological basis for the resistance to apoptosis conferred by engagement of variant CD44 by colon carcinoma cell lines. For potential relevance to the chemotherapeutic resistance frequently seen in colon cancer, we used chemotherapeutic drugs as the means of inducing apoptosis. We have found that a monolayer morphology of LIM 1863 could be induced by monoclonal antibodies directed against some epitopes of CD44, but not against others, including the HA-binding domain. The cells forming a monolayer on anti-CD44 were significantly protected from the effects of the chemotherapeutic drug BCNU. Similar protective effects of CD44 engagement were demonstrated for another CD44v6-expressing colon carcinoma cell line, but this was not the case for a colon carcinoma cell line expressing predominantly the CD44v5 isoform. Upon investigating the signaling pathways induced by variant CD44 ligation, we have defined a role for lyn protein PTK, one of the src-family of PTKs. Our results revealed that the lyn doublet was recruited to a signaling complex and activated after CD44v6 ligation, the first such demonstration in epithelial cells. Finally, we determined that the underlying mechanism of chemotherapeutic resistance required the sustained activation of the PI3K/Akt survival pathway.

Overall, the findings that variant CD44 in colon carcinoma can trigger distinct signaling pathways leading to a chemotherapeutic resistance, including activation of PI3K/Akt, may have important biological implications for an understanding of both drug resistance in tumors, as well as the selective advantages conferred to cells expressing this receptor.

MATERIALS AND METHODS

Cell Culture. LIM 1863 cells (28) were routinely grown in RPMI 1640 (Trace Biosystems, Sydney Australia) supplemented with 5% FCS (Trace Biosystems) at 37°C in a 5% CO₂ atmosphere. Low calcium growth conditions were performed in a modified calcium-free RPMI 1640 (Trace Biosystems) + 1% FCS. This medium contains ~35 μM calcium derived from the FCS (29). Intact organoids were disaggregated to a single cell suspension by culturing in this calcium-depleted medium for 1 day and then passing through a 19-gauge needle to completely separate loose clusters. LIM 1215 (30) and SW480 colon carcinoma cells were grown in DMEM (Commonwealth Serum Laboratories, Parkville, Australia) supplemented with 10% FCS.

Antibodies. The monoclonal antibody 3E8 directed against CD44 (31) was kindly provided by Dr. Graeme Russ (Queen Elizabeth Hospital, Woodville, South Australia). The anti-CD44 monoclonals BU52 and BU75 were purchased from Ancell Corp. (Bayport, MN). The anti-hCD44 and anti-hCD44v6 were obtained from R&D Systems (Minneapolis, MN). Dr. Michael Berndt (Baker Institute, Melbourne, Australia) provided the AK6 (anti-P-selectin) and AK7 monoclonals (anti-integrin α2β1), and Dr. Andrew Boyd (Queensland Institute of Medical Research, Brisbane, Australia) provided the IE8 (anti-CD36). Monoclonal and polyclonal antibodies directed against lyn, the polyclonal anti-hck antibodies, and the antiphosphotyrosine antibody PY-20 were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Akt and phospho-Akt (Serine 473) antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). The anti-α-tubulin monoclonal was purchased from Sigma Chemical Co. (St. Louis, MO), and the anti-p38 (PAb1801) monoclonal was purchased from Oncogene Science (Uniondale, NY).

Adhesion Assays. As reported previously, when single LIM 1863 cells produced by culture in calcium-depleted medium are resuspended into tissue culture medium containing calcium, reformation of the three-dimensional organoid structure occurs within 24 h (32). Alternatively, ligation of CD44 on these single cell suspensions through immobilized anti-CD44 antibody results in adherence and spreading of the cells, such that a monolayer morphology is produced over the same period instead (18). Briefly, single cell suspensions of the LIM 1863 organoid cell line were produced as described above in low-calcium media. Typically, 1.5 × 10⁵ single cells were seeded into wells of a 96-well microtiter plate into RPMI medium fully supplemented with calcium. When required, wells were precoated by incubation with monoclonal antibodies (50 μg/ml in PBS) at 4°C overnight or 1 h at 37°C. Wells were washed twice in tissue culture media before the addition of the cell volume.

For the herbimycin experiments, low-calcium suspensions of LIM 1863 cells were cultured overnight before the adhesion assay in 5 μg/ml herbimycin. Cells were maintained in the continuous presence of herbimycin at the same concentration during the adhesion assay and then visualized by light microscopy.

Drug Assays. The chemotherapeutic drug BCNU, commercially known as Carmustine (Bristol-Myers Squibb Pharmaceuticals), was reconstituted from a lyophilized dosage designed for patient administration. The stock solution was dissolved in sterile absolute ethanol to a final concentration of 50 mg/ml and subsequently diluted in tissue culture media to 1 mg/ml before use. Assays were performed in 96-well microtiter plates with a final cell concentration of 1.5 × 10⁴ cells/well, as described above. The adherent SW480 cell line was used at a concentration of 3 × 10⁴ cells/well, as these cells are relatively more spread in comparison. For monolayer formation, single cell suspensions of LIM 1863 cells were seeded onto either anti-CD44 monoclonal antibody for 24 h. Cells were cultured as suspensions or CD44-ligated monolayers (allowing even drug delivery) for an additional 24 h in the presence or absence of BCNU at the required concentration. Cell viability was assessed using an MTT assay, in which MTT (0.5 mg/ml final concentration) was added to the wells for 15–30 min, before dissolution in DMSO, and its absorbance read at 570 nm.

Immunoprecipitations and Immunoblotting. Cells were lysed in a Triton-X lysis buffer (1% Triton-X, 50 mM Tris, 150 mM sodium chloride) containing protease inhibitors (“Complete” protease inhibitor cocktail tablets; Boehringer Mannheim) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate) for 1 h. Immunoprecipitations and immunoblotting were carried out, essentially as described previously (18). Nitrocellulose filters were incubated with primary antibodies at a final concentration of 1 μg/ml for 1 h, with the exception of the Akt and phospho-Akt antibodies, which were incubated overnight at 4°C. Blots were developed using enhanced chemical luminescence. Membranes were stripped [62.5 mM Tris (pH 6.7), 100 mM β-mercaptoethanol, 2% SDS] for 30 min at 60°C before subsequent blocking and reprobing.

Transfection. Three lyn kinase constructs were used in this study, all kindly provided by Dr. Margaret Hibbs (Ludwig Institute, Melbourne, Australia). These were wild-type lyn in the pCDNAI Amp expression vector, Lyn Y397F in pCDNAI Amp (dominant negative), and Lyn Y508F in pCDNAI Amp (dominant active). The two mutant constructs contained a phenylalanine substitution for tyrosine at the amino acid positions indicated. SW480 colon cells were transfected using the electroporation method, with 65 μg DNA/reaction. Parallel transfections using green fluorescent protein were carried out to assess transfection efficiencies.

PI3K Inhibitor Assay. Single LIM 1863 cells were seeded onto immobilized anti-CD44 antibodies BU52 or 3E8 in a 96-well plate for 24 h such that the monolayer morphology was produced. Wortmannin (200 nm) and LY294002 (1 μm) were added for 2 h or the carrier DMSO as a control. At this point, additional wells were harvested for immunoblot analysis with the Akt antibodies. BCNU (100 mg/ml) was added to half the wells for a further 8 h and the wells photographed under light microscopy.

IVK Assays. Single LIM 1863 cells (5 × 10⁵) were seeded onto 6-cm plates coated with BU52 or AK7 for 45 min, which allowed attachment to both monoclonals, and lysis was performed directly on the plate. Lys immunoprecipitates were prepared as described above and washed with 200 μl of freshly prepared IVK assay buffer (50 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM phenylmethylsulfonyl fluoride, and 2 mM DTT). The kinase assay was performed by incubating the washed beads with 40 μl of IVK buffer, 10 μCi of [γ-32P]ATP, and 5 μg of enolase for 30 min at 30°C. Enolase, used as the exogenous substrate, was denatured before its addition. Briefly, 50 μg of protein was recovered from ammonium sulfate suspension by centrifugation and mixed with 5 μl of fresh IVK buffer for 30–60 min on ice. After the addition of 5 μl of glycerol, 2 μl of this solution were mixed with an equal volume of 50 mM acetic acid for 5 min at 30°C. Two μl of this solution (5 μg of denatured protein) was used in each IVK reaction. Equal loading of enolase
was confirmed after autoradiographic exposure by Ponceau S staining of the nitrocellulose membranes.

RESULTS

Variant CD44 Engagement Protects against the Chemotherapeutic Drug BCNU. We have reported previously that ligation of CD44v6 by the monoclonal antibody 3E8 induced a monolayer morphology in the spheroid colon carcinoma cell line LIM 1863 (18). To determine whether specific domains of CD44 were required to initiate this process, commercial antibodies were tested for their ability to induce the same morphological changes. The anti-CD44 antibody BU75 failed to promote monolayer formation, despite recognizing and being able to precipitate CD44 from these cells (data not shown). This antibody is known to block HA binding (33), and it is of interest that the CD44 variant in LIM 1863 fails to bind HA (18). These observations suggest that specific regions of CD44 must be engaged to promote monolayer formation by this cell line and that this region lies outside the HA-binding domain. Indeed, ligation of CD44 by the monoclonal antibody BU52, which recognizes a distinct epitope of the molecule (34), produced a monolayer indistinguishable from that induced by 3E8 (Fig. 1A).

Previous studies indicated that cells in this CD44-crosslinked monolayer were resistant to apoptosis mediated by a specific αv-integrin antibody (18). We now sought to determine whether CD44 engagement may confer resistance against other apoptotic triggers, specifically chemotherapeutic drugs. The overall sensitivity of the LIM 1863 colon carcinoma cell line to a number of chemotherapeutic drugs was tested. Drug assays used single cell suspensions of LIM 1863 cells, generated by calcium deprivation, to eliminate potential problems of drug delivery to intact LIM 1863 organoids. Calcium was subsequently replaced at the same time as drug addition. The most effective chemotherapeutic was found to be BCNU, a member of the nitrosourea family of antineoplastic agents, which killed LIM 1863 cells in a dose-dependent manner (Fig. 1). As can be seen in Fig. 1A, light microscopy showed a drug-dependent inhibition of organoid reformation, and this was found to be because of the concomitant decrease in cell viability. Typically, at the highest dose tested (100 μg/ml), 95% of the cells were dead within 24 h (Fig. 1B). The drug was also administered to LIM 1863 cells, which had formed a monolayer, after ligation of CD44 by the monoclonal antibody BU52. These cells were significantly resistant to the cytotoxic effect of BCNU over the same dose range. Visually, there was little disruption of the monolayer (Fig. 1A), and cell viability remained 90% as assessed by the MTT assay (Fig. 1B). Moreover, a second anti-CD44 monoclonal antibody tested (3E8) produced virtually identical protection from BCNU-induced loss of viability (Fig. 1C).

To determine whether this protective effect was cell line-specific, a second colon carcinoma cell line was tested. The LIM 1215 cell line grows as a typical monolayer and, like LIM 1863, expresses CD44v6 with no standard CD44 (data not shown). The LIM 1215 cell line was also sensitive to BCNU, and ligation of CD44 by either BU52 or 3E8 again offered protection against BCNU in these cells (data not shown). Thus, CD44-induced chemotherapeutic protection may be a more general mechanism for colon carcinoma cells, and whether this might relate to expression of variant CD44 isoforms is considered further below.

Ligation of Variant CD44 Activates Lyn PTK. Largely on the basis of studies in lymphoid cells, there has been a considerable amount of data to suggest that CD44 functions as an important signaling molecule. Although possessing no intrinsic kinase activity

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isoforms, p53lyn and p56 lyn, attributable to alternative splicing (39, Fig. 2, right panel). Lyn is expressed in cells as a doublet of two triplet perfectly comigrated with the two isoforms of the kinase lyn family members, where it was found that the lower two bands of the was subsequently reprobed with antibodies directed against src-PTK line (data not shown). This increase in activity was detectable within when phosphotyrosine profiles were generated for the LIM 1215 cell line (data not shown). A CD44-dependent increase in tyrosine phosphorylation was observed on a protein with identical electrophoretic mobility to the 53-kDa isoform of lyn kinase. Relative molecular masses are shown to the left in kDa.

itself, CD44-mediated signaling has been reported to involve tyrosine phosphorylation (35), including the activation of members of the src-PTK family (36–38). Therefore, we investigated if CD44 ligation in the LIM 1863 cell line led to tyrosine-dependent signaling. Single LIM 1863 cells were seeded onto either an anti-CD44 (3E8) or a control antibody over a 2-h time course, during which time the CD44-ligated cells attach and begin to spread to form a monolayer. The control antibody, AK7, also induces cellular attachment during this period via the integrin α2β1, which is eventually overcome as organoid reformation proceeds. After this 2-h period, the cells were lysed, and the lysate was immunoblotted for phosphotyrosine. The phosphoprofile obtained (Fig. 2) could be divided into two main areas: (a) a heavily tyrosine-phosphorylated region between 100 and 140 kDa, which contains such signaling molecules as p125 FAK and p130cas, and (b) a triplet of proteins between 50 and 60 kDa, correlating in mobility with several src-family kinases. There were no changes from the single cell control in tyrosine phosphorylation evident in the phosphoprofile when LIM 1863 cells were seeded onto the anti-integrin antibody. In contrast, there was a distinct elevation in tyrosine phosphorylation on the lowest phosphoprotein of the src-region triplet after ligation of CD44. Similar results were obtained when phosphotyrosine profiles were generated for the LIM 1215 cell line (data not shown). This increase in activity was detectable within 30 min, indicating that CD44 was transducing intracellular signals, even before the mechanistic event of spreading occurred. The filter was subsequently reprobed with antibodies directed against src-PTK family members, where it was found that the lower two bands of the triplet perfectly comigrated with the two isoforms of the kinase lyn (Fig. 2, right panel). Lyn is expressed in cells as a doublet of two isoforms, p53YN and p56YN, attributable to alternative splicing (39, 40). These data were consistent with the possibility that lyn kinase may be activated in response to CD44 stimulation. This was of particular interest given that lyn kinase, in addition to being implicated in CD44 signaling, has been shown previously to play a key role in the survival of hematopoietic cells (41–44).

To substantiate this finding, we used communoprecipitations to determine whether CD44 and lyn were physically associated in a signaling complex (Fig. 3). In the first set of experiments (Fig. 3A), CD44 was immunoprecipitated from single LIM 1863 cells, intact organoids, or anti-CD44 (BU52)-induced cells. These precipitates were then immunoblotted with anti-lyn antibody. As can be seen, the lyn doublet effectively coprecipitated only in those cells that were CD44 stimulated. It appears, therefore, that activation of variant CD44 induces a physical association with lyn kinase that is not constitutively present in either organoids or single cell suspensions of this line. To confirm this, the reverse experiments were also carried out, in which lyn immunoprecipitates were probed with a blotting CD44 antibody (Fig. 3B). CD44 coprecipitated specifically with lyn only in those cells that were activated through CD44. We further investigated if triggering of CD44 in the LIM 1863 cell line led to the activation of lyn kinase in IVK assays using enolase as a substrate. Single LIM 1863 cells were again seeded onto either anti-CD44 (BU52) or the control (AK7) antibody for 45 min before being harvested. As shown in Fig. 3C, cells that were triggered through CD44 displayed a consistent increase in lyn kinase activity.

To examine if this lyn activation is important in CD44-directed signaling, we used the tyrosine kinase inhibitor herbimycin, a src-family antagonist known to abrogate lyn function (45). This agent was found to prevent monolayer formation by LIM 1863 cells that had been pretreated overnight before seeding onto anti-CD44 monoclonals and tested in the constant presence of inhibitor for a subsequent 24-h period (data not shown). In contrast, herbimycin treatment had no inhibitory effect on organoid reformation by the cells seeded into suspension or on a control antibody (data not shown). These data thus

Fig. 2. CD44 ligation in LIM 1863 cells results in tyrosine phosphorylation of a protein with similarity to the PTK lyn. Single cell suspensions of LIM 1863 (sc) were seeded onto the anti-CD44 monoclonal antibody 3E8 (α-CD44) or onto the anti-integrin antibody AK7 (Control) and harvested after 30, 60, and 120 min. Lysates were immunoblotted with the antiphosphotyrosine antibody PY-20 (anti-PY, left panel), then reprobed with a monoclonal directed against lyn kinase (anti-lyn, right panel). The electrophoretic mobility of the two lyn kinase isoforms are shown (arrowheads). A CD44-dependent increase in tyrosine phosphorylation was observed on a protein with identical electrophoretic mobility to the 53-kDa isoform of lyn kinase. Relative molecular masses are shown to the left in kDa.

Fig. 3. Lyn kinase is recruited to a signaling complex and activated after CD44 ligation in LIM 1863 colon carcinoma cells. A, coprecipitation of CD44 and lyn kinase in LIM 1863 cells. Single cells, intact organoids or cells bound to the anti-CD44 antibody BU52 for 1 h (CD44 ligated) were immunoprecipitated with anti-CD44 monoclonal antibodies or an isotype-matched anti-p53 monoclonal (Control) before immunoblotting with a polyclonal antibody directed against the PTK lyn. The lyn kinase doublet (arrowhead) coprecipitated only in the CD44-ligated LIM 1863 cells. In B, in the reverse direction, antibody precipitates (or anti-Hck PTK, Control) were immunoblotted with the anti-CD44 antibody anti-CD44H, coprecipitation of CD44 (arrowhead) with lyn was only seen in CD44-ligated cells. Relative molecular masses are shown to the left in kDa. C, IVK assay of LIM 1863 cells, seeded onto the anti-CD44 antibody BU52 (α-CD44) or the anti-integrin antibody AK7 (Control) for 45 min, using enolase as an exogenous substrate. The increase in lyn kinase activity after CD44 ligation was seen in three of three experiments. Immunoblotting confirmed equal lyn protein levels in each set of precipitations, and equal enolase levels were visualized by Ponceau S staining (data not shown).
quences. To test if lyn activation and drug resistance were features of CD44-signaling cascade.

suggest that src kinases, specifically lyn, reside downstream of a selective association with the any CD44-signaling cascade (Fig. 4). This result is consistent with the possibility that both lyn signal-

Standard CD44 Did Not Elicit the Same Functional Consequences. To test if lyn activation and drug resistance were features of any CD44-signaling cascade (versus selective association with the variant isoform, CD44v), we examined another colon carcinoma cell line, SW480. This adherent cell line typically grows as a monolayer in culture and displays high levels of surface CD44 (Fig. 4A). However, unlike the LIM 1863 and LIM 1215 lines, these cells predominantly express the standard CD44 isoform (CD44s) and less abundant variant isoform(s) (CD44v) are shown by arrowheads. Relative molecular masses are shown to the left in kDa. In C, SW480 cells were seeded onto either untreated (Plastic) or immobilized-anti-CD44 (3E8) antibody-coated wells for 24 h. Increasing concentrations of BCNU were added for an additional 48 h. Viability was assessed using the MTT assay. The results represent the average of six replicates (Burr, SD).

Lyn Kinase Involvement in the Chemotherapeutic Protective Phenotype. A direct functional requirement for lyn kinase in the drug-resistant phenotype of the LIM 1863 cell line was difficult to assess because of poor transfectibility of the cells. Instead, we undertook a “gain of function” approach by transfecting the SW480 colon carcinoma cell line described above with various lyn constructs in an attempt to induce chemoresistance to BCNU. Three constructs were used: (a) a wild-type lyn, (b) a dominant-negative mutant (Y397F) in which the tyrosine at position 397 was changed to phenylalanine, and (c) a dominant-active construct (Y508F) containing a phenylalanine for tyrosine substitution at amino acid position 508 (46).

Transfections of SW480 cells with the dominant-negative lyn construct led to consistently lower (~50%) numbers of viable cells at 48 h, the time point at which the cells were harvested for immuno-

Fig. 4. SW480 colon carcinoma cells express abundant levels of CD44s and are not protected from BCNU after ligation of CD44. In A, flow cytometry with the anti-CD44 monoclonal 3E8 reveals abundant surface expression of the CD44 receptor on SW480 cells (solid plot). The monoclonal AK6 was used as a negative control (—). B, immunoprecipitation of surface biotinylated SW480 cells with the anti-CD44 monoclonal 3E8 (CD44) or a negative control antibody I8 (Control). The positions of the highly expressed standard CD44 isofom (CD44s) and less abundant variant isoform(s) (CD44v) are shown by arrowheads. Relative molecular masses are shown to the left in kDa. In C, SW480 cells were seeded onto either untreated (Plastic) or immobilized-anti-CD44 (3E8) antibody-coated wells for 24 h. Increasing concentrations of BCNU were added for an additional 48 h. Viability was assessed using the MTT assay. The results represent the average of six replicates (Burr, SD).

Data not shown). Significantly, seeding of these cells onto the anti-CD44 antibodies was carried out on single LIM 1863 cells, which were either allowed to reform organoid structures in suspension (Fig. 6A), seeded onto the control (AK7) antibody (Fig. 6B), or induced to form

Fig. 5. Chemoresistance to BCNU is induced in SW480 colon carcinoma cells after overexpression of dominant-active lyn kinase. A, lyn immunoblot of SW480 cell lysates 48 h after transfection with a wild-type lyn construct, a dominant-negative Y397F mutant, or the dominant-active Y508F plasmid. Endogenous lyn kinase levels are shown in mock-transfected cells. In B, SW480 colon carcinoma cells were transiently transfected for 48 h, seeded into microtiter wells either uncoated (Plastic) or coated with immobilized 3E8 monoclonal antibody (anti-CD44), and allowed to adhere overnight. BCNU (100 μg/ml final concentration) was added for 24 h, and viability was assessed using the MTT assay. Results shown are from triplicate experiments (Burr, SD).
a monolayer after ligation of CD44 (Fig. 6C). Levels were assessed at both 6 and 24 h time points, the latter corresponding to the time of BCNU addition to the monolayers and, hence, the functionally relevant time point.

In all cases, there was an elevation of phosphorylated Akt at the 6-h time point, most likely reflecting a serum-induced effect. However, in the cells reforming organoids in suspension and on the control antibody, Akt activity was significantly reduced by 24 h, as evidenced by loss of phosphorylated Akt (and a mild reduction in protein levels). In contrast, cells of the CD44-ligated monolayer maintained elevated levels of phosphorylated Akt at 24 h (Fig. 6C). This sustained Akt activation was also measurable by Akt-IVK assays using glycogen synthase kinase-3 as a substrate (data not shown). Sustained Akt activation in these colon cells in response to CD44 ligation would be consistent with a role for Akt in the chemoresistant phenotype.

To substantiate a functional role for Akt activation in the drug resistance of the CD44-mediated monolayer, PI3K inhibitors were used to block Akt activation. In so doing, we wished to test whether these agents also blocked the protective phenotype of the CD44 signaling against BCNU (Fig. 7). A 2-h pretreatment with the PI3K inhibitor Wortmannin completely inhibited phosphorylation of Akt, without affecting the level of expressed protein (Fig. 7A). Continued Wortmannin treatment (or the carrier alone as a control) had no detectable effect on the monolayer cells in the absence of chemotherapeutic drug (Fig. 7B). However, in contrast to the carrier-treated controls, Wortmannin-treated cells were highly sensitive to the addition of BCNU, with extensive rounding up and detachment of dying cells within 8 h (Fig. 7B). Similar results were obtained using a second pharmacological inhibitor of PI3K, LY294002. This reversal of CD44 protection by both PI3K inhibitors was observed on monolayers produced by two anti-CD44 monoclonals (data not shown). Taken together, these data establish that the continued activation of the PI3K/Akt survival pathway, induced through the ligation of variant CD44, is required for the chemotherapeutic resistance.

Finally, we investigated if there was a link between lyn activation and Akt elevation in the colon carcinoma cells. SW480 cells were transfected with either wild-type or dominant-active (Y508F) lyn and harvested 48 h after transfection. Akt activity was assessed by immunoblotting the cellular lysates with phospho-Akt and anti-Akt antibodies. As shown in Fig. 8, Akt activity was indeed stimulated in those cells expressing the constitutively active lyn mutant, without any increase in protein levels, but not in the mock- or wild-type lyn-transfected cells. This result demonstrates that activated lyn kinase is sufficient to promote sustained activation of Akt. Furthermore, because these SW480 cells do not spread as a result of transfection, this result argues against effects of adherence and spreading as being responsible for Akt activation and survival.

DISCUSSION

In this study, we provide the first evidence that a variant of the cell surface receptor CD44 can promote drug resistance in tumor cells. In two colon carcinoma cell lines, LIM 1863 and LIM 1215, engagement of CD44v6 by two independent monoclonal antibodies led to a chemotherapeutic-resistant phenotype. In preliminary experiments, we found that drug sensitivity was not uniform between both cell lines. E.g., whereas LIM 1215 cells were susceptible to 5-Fluorouracil, the most commonly used drug in the clinical treatment of colon cancer, LIM 1863 cells were highly resistant to the cytotoxic effects of this drug, if used alone or in combination with leucovorin. However, both cell lines showed sensitivity to BCNU, one of the nitrosourea family of antineoplastic agents. This class of drugs consists of alkylating

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agrees that agents which are effective in all phases of the cell cycle. Monolayer formation, induced by either monoclonal BU52 or 3E8, resulted in profound resistance to the cytotoxic effects of this agent in the two colon carcinoma cell lines (Fig. 1). Additional studies will be required to determine whether this, or other, CD44 variant molecules can offer protection against multiple drugs in a variety of tumor types. However, it is interesting to note that nitrosoureas are also used in the treatment of non-Hodgkin’s lymphoma (nitrogen mustard-vincristine-procarbazine-prednisone therapy), another tumor type for which high CD44v6 expression is an indicator of poor prognosis (49).

Traditionally, antibody studies have been used to identify and characterize cell adhesion molecules, as certain antibodies may influence adhesive functions or are informative about ligand-binding sites (50). Using such an approach, functional regions of CD44 have now been identified, in particular, the site responsible for HA binding (50). In a recent study using a CD44/Fas chimera, Ishiwarti-Hayasaka et al. (51) showed that antibody ligation of a specific region of the CD44 extracellular domain, outside the HA-binding region, resulted in signal delivery. We were initially interested in which regions of CD44 were activated to induce a monolayer morphology in the LIM 1863 spheroid cell line, a phenomenon we had defined in a previous study with the monoclonal antibody 3E8 (18), the epitope of which is not known. Thus, we tested additional antibodies for their ability to induce the same extensive morphological changes. One antibody, BU75, which blocks HA binding (33), failed to induce a monolayer in these cells. In contrast, we could overcome the intercellular adhesive forces of the cell line and induce a monolayer through ligation of CD44 using the monoclonal antibody BU52. The epitope bound by BU52 has been localized to an area on the disulfide-bonded NH2-terminal region of CD44, outside the HA-binding site (34). Further, we have shown that the CD44v6 variant in this line fails to bind HA (18). Taken together, these data indicate that the region of CD44 that signals monolayer formation is most likely independent of the HA-binding domain.

It has emerged that tyrosine kinases play an important role in the transduction of regulatory signals by CD44 in a number of cell types. The first clear demonstration of this was by Taher et al. (36), who provided evidence of a functional and physical association of the src-family member p56^{lck} with standard CD44 in T cells. Subsequently, tyrosine kinase activity after CD44 activation has been shown for natural killer cells (35), to involve the PTKs lyn and hck in human neutrophils (37), and with complex formation of lck and lyn in murine T and B cells, respectively (52). Even so, it is still unclear how CD44 interacts with src-family members. There is evidence for a possible direct interaction for lck and lyn with a region corresponding to the plasma membrane-cytoplasmic interface in murine CD44 (52). Alternatively, the ankyrin binding region of the cytoplasmic tail of CD44s has been shown to be necessary for an association with src kinase itself in prostate tumor cells (38), indicating that a multimolecular complex may be formed.

In apparent agreement with these earlier studies examining standard CD44, we have shown that ligation of variant CD44 in the colon carcinoma cells resulted in the activation of lyn kinase, further implicating tyrosine kinases as central effectors in CD44-mediated signaling cascades. However, differences were seen, and given the role of variant CD44 isoforms in tumor progression, these may eventually have important implications for tumor biology. The CD44s-lyn association described previously for both unstimulated murine B cells (52) and in human neutrophils (37) was shown to be a stable, constitutively present interaction, and no alteration in lyn (or hck) activity was detected after incubation with anti-CD44 antibodies used in the neutrophil study (37). In contrast, our data in colon carcinoma cells revealed that the CD44v6-lyn interaction was detectable only after CD44 ligation. Therefore, the results suggest that, upon activation of CD44v6 by the immobilized anti-CD44 antibody, lyn kinase is recruited into a signaling complex. Whether this lyn recruitment is a feature of the variant isoform remains to be determined.

We have shown for the first time that ligation of CD44, and in particular a variant isoform, can lead to up-regulation of PI3K/Akt activity. A recent study by Gaumet et al. (53) may be of relevance to the findings we are reporting here. They showed that the plasma membrane-microfilament linker ezrin, one of the ERM family of proteins, could interact with the p85 regulatory subunit of PI3K in an epithelial cell line. Further, a point mutation of ezrin abrogated this interaction, impaired Akt activation, and induced apoptosis. Taken together, their data suggested a function for ezrin in promoting cell survival, involving the PI3K/Akt pathway. The ERM protein family functions in part as a cross-linker between the actin cytoskeleton and the plasma membrane. These proteins contain an actin-binding domain toward the COOH-terminal end and a conserved FERM domain near the NH2 terminus that interacts with several transmembrane adhesion molecules, including CD44 (54, 55). Thus, a CD44-ezrin interaction could represent a possible mechanism that is involved in regulating the sustained Akt response. Moreover, the CD44 cytoplasmic domain displays a relatively weak binding for ERM proteins under physiological ionic strength conditions (56), indicating that ERM proteins may need to be activated to function as cross-linkers. It has been proposed that the cytoplasmic domain of CD44 interacts with different cytoskeletal proteins with different binding affinities (e.g., a comparatively high binding affinity for ankyrin; Ref. 57) and that these differential binding affinities may influence selective signaling pathways leading to the onset of different CD44-mediated functions (6). Clearly, the possible functional consequences of the lyn kinase activation we have found is not inconsistent with this hypothesis.

Is the lyn kinase activation by CD44 a requirement for a chemotherapy-resistant phenotype? Lyn recruitment and activation after CD44 ligation appears necessary for monolayer establishment in the LIM 1863 cells, as evidenced by the herbimycin result. However, because of the poor transfectability of these cells, it was difficult to assess any downstream functional consequences for survival by lyn in this line. Instead, we used a gain-of-function approach in a “CD44-unresponsive” colon cell line, SW480. Ligation of CD44 on these cells by our anti-CD44 monoclonal antibodies failed to elicit lyn activation nor, most importantly, did they promote any resistance to BCNU. We concluded that this was attributable to these cells predominantly expressing the standard form of CD44. However, che-
more resistance to BCNU was induced in these cells by overexpression of dominant-active lyn kinase, thereby implicating a role for lyn in triggering the biological pathways necessary for the chemoresistant phenotype. These findings, along with the demonstration that Akt was activated in these cells (see below), strongly suggest that lyn kinase is an essential mediator of CD44-directed chemoresistance in colon carcinoma. As such, this is the first demonstration of lyn-kinase-dependent regulation of cell survival in epithelial cells. Lyn function has been largely characterized in hemopoietic cell lineages, where critical roles in mediating cell survival have been described. These include lyn activation as being essential for the antiapoptotic effect of granulocyte macrophage colony-stimulating factor and IL-5 in eosinophils (43, 44), in playing a key role in preventing neutrophil death via granulocyte macrophage colony-stimulating factor signaling (42), and in regulating apoptosis through its association with the CD19 surface receptor in B cells (41). Additional lines of evidence have supported the conclusion that lyn kinase may be important for the regulation of PI3K. Activation of the src-family of kinases, including lyn, is accompanied by a conformational change exposing the src homology 3 domain (58), which has been shown to bind to the proline-rich regions of the p85 subunit of PI3K (48). In addition, lyn had been reported previously to associate with PI3K after BCR-mediated signaling (47).

Lyn regulation of Akt, however, is somewhat more controversial. Recently, the BCR was shown to activate Akt, via PI3K, and this was proposed to be completely dependent on lyn kinase, as Akt was not phosphorylated in response to BCR engagement in lyn-deficient DT40 cells (59). However, another study reported that BCR cross-linking in the same lyn-deficient B cells resulted in enhanced phosphorylation and activation of Akt, suggesting that lyn may negatively regulate Akt function (60). Similar findings of an antagonistic role for lyn in BCR-induced Akt activation have also been reported by others (61). The reason for this discrepancy in apparently similar cell types is presently unclear, and therefore, these data need to be interpreted with caution. Further, in each of these studies, the authors showed a requirement for Syk kinase in mediating the Akt response, and, given that Syk is a known target of lyn kinase, there may exist some functional redundancy in the BCR-signaling pathway. Consistent with this, both Syk and lyn deficiency have been shown to enhance ceramide-induced apoptosis in the DT40 cell line (62). Significantly, our results have established a direct link between lyn activation and increased Akt activity in colon carcinoma cells. As shown by the transfection studies in the SW480 carcinoma line, expression of a constitutively active lyn kinase was sufficient to promote the sustained activation of Akt. It is reasonable to suggest, therefore, that there exists a number of alternative signaling pathways which ultimately lead to Akt elevation, and we are currently investigating this possibility in our colon carcinoma model system.

In summary, our results establish a novel function for CD44 in determining the survival of colon carcinoma cells by inducing chemotherapy resistance. Further, they suggest that variant CD44 isoforms can activate distinct signaling pathways in response to external cues. As such, these findings may be of some biological significance and point toward the selective advantages imparted by variant CD44 expression. We further suggest that the development of an inherent ability to suppress apoptosis might play a critical role during colorectal tumorigenesis.

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A CD44 Survival Pathway Triggers Chemoresistance via Lyn Kinase and Phosphoinositide 3-Kinase/Akt in Colon Carcinoma Cells


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