Collagen I Provides a Survival Advantage to MD-1483 Head and Neck Squamous Cell Carcinoma Cells Through Phosphoinositol 3-Kinase Signaling

ERIK COHEN1,2, TARA TENDLER2,3, HAIYAN LU2,3, CHRISTOPHER K. HANSEN2,3, JULIAN KURTSMAN2,3, JUDITH BARRIOS2,3 and ROBERT WIEDER2,3

Departments of 1Otolaryngology-Head and Neck Surgery and Medicine and the 3New Jersey Medical School-University Hospital Cancer Center, University of Medicine and Dentistry of New Jersey, Newark, NJ, U.S.A.

Abstract. Background: Head and neck squamous cell carcinoma (HNSCC) has a 50% relapse rate. The tumor microenvironment has been linked to resistance of cancer cells to chemotherapy. We hypothesized that the tumor matrix proteins collagen and fibronectin play protective roles in HNSCC. Materials and Methods: We investigated the effects of collagen I, collagen IV and fibronectin on growth, 2-D and 3-D clonogenic potential, resistance to paclitaxel, apoptosis and activation of phosphoinositol-3 kinase (PI3K) in MD-1483 HNSCC cells. Results: Collagen I, collagen IV and fibronectin specifically increased the efficiency of 2-D colony formation through binding integrins α2β1 and α5β1, respectively, and provided resistance to paclitaxel-induced colony elimination and apoptosis. Collagen I, but not fibronectin, increased the efficiency of 3-D colony formation and induced resistance to paclitaxel. Activation of protein kinase-B by collagen I was necessary for the protective effect. Conclusion: These data support the potential contribution of fibronectin and collagen to chemotherapy resistance in HNSCC, with effects of collagen mediated by PI3K.

Squamous cell carcinoma of the head and neck (HNSCC) requires multimodality therapy and frequently frustrates attempts at long-term success. About half the patients with HNSCC experience disease recurrence after initial therapy. Recurrent disease is resistant to treatment and overall five-year survival remains at about 50% (1). Factors with described roles in treatment resistance include multiple signaling pathways, resistance mechanisms, inflammation, the microenvironment, and the presence of cells with intrinsic resistance and a capacity to repopulate the tumor in all its heterogeneity after all other cells were eliminated.

Epidermal growth factor receptor (EGFR)-initiated signaling, is perhaps the best defined pathway with known roles in treatment resistance. EGFR is frequently overexpressed in HNSCC and is associated with disease progression, chemotherapy resistance and reduced survival (2, 3). Mechanisms of EGFR-induced aggressive behavior are mediated through the phosphoinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (4, 5) and up-regulation of cyclin D1 (6, 7). Therapy directed at EGFR-initiated signaling has only modest activity due to EGFR and RAS mutations, epithelial-mesenchymal transition (EMT) (3, 8, 9), modulation of Kruppel-like factor-4 (10), and activation of alternate pathways through mutations in p53 (11) and proteins that regulate expression of p53 target genes (12).

Response to DNA-damaging therapy in HNSCC is linked to increased DNA repair and telomere maintenance response by the meiotic recombination 11 homolog /Rad50 homolog/nijmegen breakage syndrome 1 protein (MRN) complex (13). High expression of excision repair cross-complementation group 1 (ERCC1) and ERCC4, an essential component of several DNA repair pathways in primary HNSCC and metastases, predict a poor treatment response, EMT, early time-to-progression and poor survival (9, 14-16). Snail1 (SNAIL) directly regulates ERCC1 transcription and their coexpression correlates with cisplatin resistance and a poor prognosis (9).

The expression of multidrug-resistance (MDR) genes adenosine triphosphate-binding cassette transporter and P-glycoprotein are independent prognostic factors and predictive for malignant progression in laryngeal squamous cell carcinoma (17). The expression of numerous drug transporters and phenotypic MDR are markedly induced by paclitaxel in HNSCC (18). Overexpression of antiapoptotic members of the breakpoint cluster-2 family also influences resistance to therapy (19-20).

Correspondence to: Robert Wieder, MD, Ph.D., 205 South Orange Avenue, Cancer Center H1216, Newark, NJ 07103, U.S.A. Tel: +1 973 9724871, e-mail: wiederro@umdnj.edu

Key Words: Squamous cell carcinoma, head and neck cancer, collagen I, phosphoinositol-3 kinase, MD-1483 cells.
Inflammation plays a significant role in resistance and poor prognosis in HNSCC. Heterogeneity among toll-like receptors (21), alterations in signaling through the transforming growth factor-β (TGFβ) pathway (22, 23), nuclear factor-kappa B activation (24, 25) and activation of cyclooxygenase 2 and derived prostaglandins, particularly prostaglandin E2 (26), have been described as contributing to treatment resistance of HNSCC.

Signal transducer and activator of transcription (STAT) pathways also have significant influence on HNSCC growth and treatment resistance (27-30). High levels of STAT3 are associated with Notch-1 signaling, a pathway required for cancer re-populating capacity and cytotoxin resistance (30). HNSCC contain a subpopulation of cells expressing CD44, the hyaluronan receptor, which can reproduce original tumor heterogeneity and can be serially passaged, characteristics of tumor-initiating cells (31). Hyaluronan promotes CD44/EGFR interaction, EGFR-mediated oncogenic signaling and chemotheray resistance, suggesting a significant role of the microenvironment in HNSCC treatment resistance (32). Because of these data and a demonstrated role of the microenvironment in other tumor types, we investigated roles and mechanisms of collagen and fibronectin-supported survival of a HNSCC cell line.

Materials and Methods

Cells and culture. MD-1483 HNSCC cells were obtained from the American Type Culture Collection (Manassas, Virginia) and maintained in Dulbecco’s Modified Eagle Medium (DMEM)/Ham F-12 supplemented with 5% fetal bovine serum, 1:1000 penicillin/streptomycin, and 1:1000 L-glutamine at 37°C with 5% CO2. For determining competitive proliferation rates, cells were incubated on tissue culture-coated dishes or dishes coated with fibronectin, collagen I, or collagen IV (BD Biosciences, San Jose, CA, USA). One day after incubation, the media were replaced with fresh media or media containing paclitaxel at 10^-8, 10^-7 or 10^-6 M. Adherent and floating cells were combined on day 1 and on day 2 after paclitaxel addition and incubated with annexin V-flourescein isothiocyanate (FITC) 1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and propidium iodide (PI) 2 μg/ml (Sigma-Aldrich, St. Louis, MO, USA) in 1× binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Early apoptosis was denoted by the percentage of cells that were annexin V-positive/PI-negative and late apoptosis by cells that were annexin V-positive/PI-positive.

Western blots. Total and phospho-Akt levels relative to controls were determined by western blot. Cells were cultured to sparse density incubated on tissue culture-coated dishes or dishes coated with collagen I, collagen IV, or fibronectin (BD Biosciences), harvested, pelleted, and lysed in RIPA buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM sodium orthovanadate] supplemented with protease inhibitor cocktail (Sigma-Aldrich). The lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the appropriate antibodies. Antiphospho-AKT (Ser 473) was purchased from Santa Cruz Biotechnology and anti-AKT from Cell Signaling Technology (Beverly, MA, USA). Complexes were visualized using secondary horseradish peroxidase-conjugated anti-rabbit (Bio-Rad Laboratories, Hercules, CA, USA) or anti-mouse IgG (Santa Cruz Biotechnology) at 1:3,000 dilution and an ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) (35). A nonspecific band on the Coomassie-stained membrane was used as a loading control.

Statistical analysis. Statistical analysis was performed using the two-sample Student’s t-test. Two-tailed p-values of 0.05 or less were considered significant.

Results

We determined the effects of the tumor stromal proteins collagen and fibronectin on the behavior of MD-1483 HNSCC cells using a variety of in vitro assays that reflect the malignant phenotype. When measuring proliferation, neither collagen I nor fibronectin had significant effects on the rate of cell growth (data not shown). Two-dimensional colony assays, which reflect a variety of factors, including adhesion, proliferation and survival, demonstrated that the clonogenic potential was considered significant.
significantly influenced by the substrata (Figure 1A). Cells incubated on collagen I, collagen IV and fibronectin had more than 100% increases in relative 2-D cloning efficiency compared to cells incubated on tissue culture-coated plates. The absolute cloning efficiency of cells varied with passage from the time of thawing from cryopreservation, as demonstrated by the variable baseline clonogenic potential reported in the different Figures. The advantage in 2-D clonogenic potential endowed by collagen I and fibronectin, once again demonstrated in Figure 1B, was specific. Blocking integrin α2β1, collagen receptor-1, significantly and specifically reversed the colony forming advantage endowed by collagen I and blocking integrin α5β1, a fibronectin receptor, significantly and specifically reversed the colony forming advantage endowed by fibronectin. Control IgG had no effect.

The clonogenic advantage endowed by the matrix proteins translated to a measurable effect under cytotoxic conditions as well. Cells exposed to paclitaxel at 10^{-9} M formed 2-D colonies at twice the efficiency when incubated on collagen I, collagen IV or fibronectin than on tissue culture-coated plates (Figure 2). Concentrations of paclitaxel of 10^{-8} M prevented colony formation under most circumstances, regardless of substrata. Since matrix proteins did not influence proliferation, we investigated other factors involved in successful colony survival. We determined the effect of matrix proteins on resistance to apoptosis induced by paclitaxel. Late apoptosis induced by 10^{-8}, 10^{-7} and 10^{-6} M paclitaxel was inhibited by both collagen I and fibronectin but with a greater impact by collagen I than fibronectin at more toxic paclitaxel concentrations (Figure 3). Early apoptosis was not influenced by substrata.

The mechanical force on cells growing on tissue culture plates is more than 10^4-fold higher than that of cells in vivo (36). Mechanical force initiates signaling that often masks significant biological effects under more physiological conditions. We therefore utilized a 3-D soft agar colony assay system with dissolved collagen I or fibronectin to determine their effects on clonogenic potential and sensitivity to paclitaxel. Collagen I significantly increased the baseline 3-D clonogenic potential of MD-1483 cells in soft agar, as well as their capacity to retain anchorage-independent colony-forming capacity in 10^{-9} and 10^{-8} M paclitaxel (Figure 4). The percent residual colony-forming potential in paclitaxel as compared to agar alone was also higher in agar containing dissolved collagen I than in agar containing media alone or fibronectin. These data demonstrate that in 3-D, collagen I but not fibronectin provides a distinct advantage in anchorage independent growth at baseline and under cytotoxic pressure from paclitaxel.

Prior studies in other systems have demonstrated that PI3K signaling, initiated by ligation of integrins provides a specific survival advantage to cancer cells (37). We evaluated the activation of the PI3K pathway by assessing the activating
phosphorylation of serine 473 of AKT, a downstream target of activated PI3K. Incubation of cells on collagen I-coated tissue culture plates induced a significant phosphorylation of AKT, as compared with tissue culture- and fibronectin-coated plates (Figure 5). The phosphorylation of AKT was diminished by the PI3K inhibitor LY294002 at 5 μM and reverted to baseline due to LY294002 at 10 μM. Based on these data, we wanted to determine the functional role of the activation of the PI3K pathway on survival of MD-1483 cells from cytotoxic exposure. We determined the influence of matrix protein-induced PI3K signaling on 2-D colony formation and resistance to paclitaxel toxicity. Paclitaxel at 6×10^{-10} M significantly inhibited the efficiency of colony formation on tissue culture-coated and collagen I-coated plates, but not on fibronectin-coated plates (Figure 6A). The effect, however, was very small at this concentration. There was no clonogenic advantage imparted to the cells by collagen I or fibronectin in this experiment, an observation that varied with cell-cloning efficiency. Interestingly, inhibition of PI3K by LY294002 at 5 μM significantly, consistently and markedly increased the colony-forming potential of the cells on all three substrata. However, when combined with paclitaxel, LY294002 caused a marked decrease in the colony numbers on all three plate coatings as compared with paclitaxel at 6×10^{-10} M alone. We noted that in addition to effects on colony numbers, chemotherapy and PI3K inhibition had significant effects on colony size. We measured colony size using an imaging program and determined that incubating cells on both fibronectin and collagen I caused significant increases in colony size compared with those on tissue culture-coated plates with media alone, as well as in the presence of paclitaxel at 3×10^{-10} M and 6×10^{-10} M (Figure 6B). While paclitaxel at these concentrations had no effect on colony size on tissue culture-coated plates (p>0.05), the colonies treated with LY294002 were significantly smaller than those from untreated cells (p<0.005). Collagen, but not fibronectin induced a significant increase in colony size in LY294002-treated cells. The inhibition of PI3K in cells treated with paclitaxel at 6×10^{-10} M completely eliminated the effects of collagen I and fibronectin in promoting increased colony size. These data demonstrate that inhibition of PI3K potentiates the effects of low concentrations of paclitaxel both on clonogenic potential and clone size and reverses the survival advantage endowed by collagen I and fibronectin.

**Discussion**

HNSCC contain an abundance of adhesion molecules, including integrins (38) and hyaluronan (39). Head and neck tumors depend on these adhesion molecules for survival through stromal interactions. For example, integrin αvβ6 promotes tumor progression, metastasis and resistance to radiation therapy through TGFβ signaling (40, 41). Integrins induce TGFβ activation in both the epithelial cells and
associated tumor fibroblasts (42), resulting in activation of inflammatory signaling (43) and increased cytokine responsiveness in HNSCC (44). Integrin ligation initiates outside-in signaling through integrin-linked kinase, which causes a variety of additional intracellular events, including the formation of focal adhesion complexes, the activation of small GTPases, of PI3K and the extracellular receptor kinase pathways, histone H3 deacetylation, reduction of DNA double strand breaks and lethal chromosome aberrations (45, 46). Blockade of integrin-linked kinase-mediated signaling, of focal adhesion kinase or of inflammatory signaling can reverse radioprotection-induced interactions with the microenvironment (45, 47, 48). The data we present here demonstrate that ligation of integrins α2β1 and α5β1 by collagen and fibronectin, respectively, provide a survival advantage to MD-1483 HNSCC cells in vitro in 2-D clonogenic assays. The effects are manifested as an absolute increase in clonogenic potential, as well as an endowment of a relative resistance to paclitaxel. Prior investigations in other tumor types have shown a survival advantage conferred by collagen (49, 50).

The 2-D culture system, however, while a useful and long-utilized model for assaying cell biology and behavior, does not adequately reflect the behavior of cells in vivo. The mechanosensory force of the tissue culture plate alone is orders of magnitude higher than that of cells, even in an indurated tumor, and can initiate signaling that can mask relevant factors that contribute to molecular and cellular effects (36). The 3-D structure of tumors and the interaction with elements of the microenvironment are important factors contributing to their survival, behavior and response to therapy (51).

Interaction of HNSCC with its microenvironment in 3-D in vitro and in vivo results in resistance to radiation-induced cytotoxicity (46, 47) and activation of hypoxia-induced factors.
through TGFβ (52). While prior studies demonstrated a survival advantage endowed by collagen and fibronectin in monolayers (37, 49), collagen was also shown to provide an advantage in clonogenic potential in anchorage-independent growth in other cancer cell types (53). We tested a 3-D clonogenic assay model with dissolved collagen I and fibronectin to confirm the effects we observed in monolayers. In 3-D, collagen but not fibronectin, provided a clonogenic growth advantage as well as a survival advantage to paclitaxel cytotoxicity. This pattern was reflected by the differential activation of PI3K by collagen but not fibronectin. The PI3K pathway is considered a therapeutic target in HNSCC, particularly since a battery of novel inhibitors are winding their way through human drug development trials (54). These data suggest that while PI3K pathway activation by collagen I may be the significant modulator of survival in 3-D, fibronectin, which also promotes survival and resistance in 2-D culture, likely activates different pathways to achieve this effect. Our data show that both matrix proteins contributed to a relative inhibition of late apoptosis when cultured in 2-D and blocking interactions with them may be a potential mechanism to accentuate the effects of chemotherapy.

Acknowledgements

Supported by a ASCO Young Investigator Award (EC), Foundation of University of Medicine and Dentistry of New Jersey (EC and RW) and the Ruth Estrin Goldberg Foundation (EC and RW).
References


6 Kothari V and Mulherkar R. Inhibition of cyclin D1 by shRNA is associated with enhanced sensitivity to conventional therapies for advanced head and neck squamous cell carcinoma. PLoS ONE 2: e26399, 2011.


