Desmoid Cell Motility Is Induced In Vitro by rhEGF

David E. Joyner, Sylvia H. Trang, Timothy A. Damron, Albert J. Aboulafia, Judd E. Cummings, R. Lor Randall

1SARCTM Laboratory, Sarcoma Services, Department of Orthopaedics and Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, Utah 84112, 2Department of Orthopaedics, SUNY Upstate Medical University, Syracuse, New York 13202, 3Sinai Hospital Cancer Institute, Baltimore, Maryland, 21215

Received 6 October 2008; accepted 28 January 2009
Published online 25 February 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20865

ABSTRACT: Desmoid tumors are benign but locally invasive myofibroblastic lesions that arise predominantly in the abdominal wall or shoulder and are prone to aggressive local recurrences. A perceived association between desmoid activity and the expression of growth factors during pregnancy or following trauma suggests a cause-and-effect relationship between growth factor stimulation and desmoid invasiveness. We used Boyden Chambers to quantify cell motility in order to determine the effect of growth factor stimulation on desmoid cell migration. Desmoid cell cultures were treated under serum-free conditions with epidermal growth factor (rhEGF) or transforming growth factor alpha (rhTGFα). Additional cell cultures were pretreated under serum-free conditions with the EGF receptor (EGFR) inhibitor AG1478, alone or in combination with the TGFβ1 receptor inhibitor SB431542, and then stimulated with growth factor prior to being assayed for cell motility. The experiments demonstrated a direct dose-dependent relationship between rhEGF stimulation and desmoid motility. In contrast, rhTGFα was less effective at inducing cell migration. rhEGF-induced cell migration could be diminished, but not reduced to control levels, by inhibiting EGFR. When EGF and TGFβ1 receptors were inhibited simultaneously, the level of rhEGF-induced cell migration was reduced significantly beyond the level of cell migration generated by inhibition of EGFR alone. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1258–1262, 2009

Keywords: desmoid tumor; growth factors; cell motility; in vitro

Desmoid tumors are locally invasive myofibroblastic lesions that arise predominantly in the abdominal wall or shoulder and are prone to aggressive local recurrences without metastases. Desmoids have been associated with trauma, hormonal activity, and genetic alterations and often develop in women during or after pregnancy, thus suggesting a cause-and-effect relationship between growth factor stimulation and desmoid activity. Genetic abnormalities frequently detected in desmoids include somatic mutations in codons 41 or 45 of exon 3 of beta catenin (CTNNB1), and germline mutations in the adenomatous polyposis coli (APC) gene of tumors associated with familial adenomatous polyposis (FAP). Less frequently reported aberrations include reduced expression of the Retinoblastoma (RB) tumor suppressor gene, upregulation of Wilms’ tumor gene 1 (WT1), and polymorphism in the plasminogen activator inhibitor 1 (PAI-1) promoter.

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are essential participants in the process of wound healing and their common receptor (EGFR) is typically upregulated in uteroplacental tissues during childbirth. Overexpression of EGFR also correlates with tumor aggressiveness in a variety of cancer types. Furthermore, EGF induces cell growth, migration, and invasiveness in cultured human glioma cells. Recently, we reported that the coexpression of 15 tumor-associated genes correlated with tumor EGF transcript content in 31 benign mesenchymal tumors (6 histotypes including desmoids) and in 42 primary sarcomas (11 histotypes). Coexpressed genes detected in the benign tumors included matrix metalloproteinase 1 (MMP1), tumor necrosis factor receptor superfamily, member 6 (PAS/CD95), tumor protein 53 (TP53), cyclin-dependent kinase inhibitor 1A (CDKN1A/p21), and B-cell lymphoma 2 (Bcl-2). Since (1) EGF induces cell motility and invasiveness in other cell types, and (2) gene expression in benign mesenchymal tumors correlates with EGF content, we proposed that EGF and/or TGFα are involved in the progression of desmoid tumors. Specifically, we hypothesized that EGF and TGFα, acting independently or interactively, mediate desmoid invasiveness via their transcriptional regulation of relevant motility genes.

MATERIALS AND METHODS

Tissue Acquisition and Processing

Sections from 18 desmoid tumors transported and maintained at 4°C in RNAlaterTM (Ambion, Austin, TX) were acquired from three institutions and processed for mRNA according to IRB approved and HIPAA compliant protocols. Total RNA was extracted and purified from the homogenized tissues using RNeasy® kits supplied by Qiagen (Germantown, MD) and RNA quality evaluated using an Agilent 2100 Bioanalyzer (Santa Clara, CA).

Desmoid Cell Lines

Three resected desmoid tumors were dissociated for 2 h at 37°C in Collagenase 1A (100 collagen digestion units/mL; Sigma C9891; St. Louis, MO). The resulting desmoid cell cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS; HyClone, Logan, UT); culture medium containing FBS is referred to hereafter as “complete medium.” The cultures were grown to near confluency in 75-cm² tissue culture flasks (cell passage #1) and then subdivided for continued in vitro culture or frozen for subsequent use. Two cell lines (SS07-008; SS07-037) were used interchangeably throughout this study to ensure the results were not cell-line specific. The third cell line (SS07-092) was cultivated through cell passage #1 in order to evaluate changes in mRNA content induced by in vitro culture. Cultures
were maintained in mid-log phase within a 37°C humidified incubator containing 5% CO2. All in vitro experiments involved cell cultures subjected to fewer than 15 cell passages.

**Real-Time RT-PCR**

Tumor and cell line mRNA content was quantified by real-time RT-PCR using TaqMan® Gene Expression Assays and the ABI PRISM® 7900 HT Sequence Detection System with related software (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference standard. The 18 tumors and three desmoid cell lines were screened for EGF, TGFα, EGFR, signal transducer and activator of transcription 3 (STAT3), integrin beta 1 (ITGB1), MMP1, and matrix metalloproteinase 3 (MMP3) mRNA content. Cell culture RNA was extracted and purified as described above.

**Boyden Chamber Motility Assays**

Cell motility assays were conducted under serum-free (SF) conditions and involved a minimum of two replicate Boyden Chamber experiments with two-to-four independent cell cultures/treatment/experiment. Desmoid cells seeded at 1.5–2.0 × 10^5 cells per 25-cm² tissue culture flask in complete medium were serum starved for 24 h, and then stimulated with 5–100 ng/mL rhEGF (E9644; Sigma) or rhTGFα (T5403; Sigma) for an additional 24 h. Control flasks received SF DMEM only. The cultures were assayed for cell motility using a colorimetric CytoSelect Cell Haptotaxis Assay Kit (CBA-100- FN; 8 μm pore, Fibronectin-coated membrane; Cell Biolabs, Inc., San Diego, CA) according to the manufacturer’s recommendations. Cell cultures were pelleted by centrifugation and resuspended in 0.5 mL of a 5% bovine serum albumin/DMEM loading buffer. Four hundred microliters of the original 0.5 mL cell suspension was then loaded into individual chambers. The residual 100 μL were used to monitor loading densities. The chambers were incubated for 3 h at 37°C prior to cell staining. The dye was extracted subsequently from cells that migrated through the fibronectin-coated membrane and quantified using a microplate reader set to 560 nm. Spectrophotometric absorbance correlates with cell migration.

To determine the affect of receptor inhibitors on cell motility, desmoid cultures were pretreated for 24 h with the EGFR inhibitor AG1478 (tyrphostin; T4182; Sigma), alone at 200, 300, or 400 nM, or in combination with the TGFβ1 receptor inhibitor SB431542 (Tocris Biosciences, Ellisville, MO). Following the initial 24 h inhibitor treatment, cultures were then stimulated for an additional 24 h with 200 ng/mL rhEGF coupled with the receptor inhibitor(s) prior to being assayed for cell motility.

**Proliferation Assay**

Desmoid cells were seeded into 25-cm² tissue culture flasks at 0.5–1.0 × 10^5 cells/flask. Following a 24 h incubation in complete medium to allow attachment, the cultures were serum deprived for 24 h, and then either (1) treated for an additional 24 h under SF conditions with rhEGF at 100–200 ng/mL and quantified, or (2) exposed to 400 nM AG1478 dissolved in methyl sulfoxide (DMSO; Aldrich, Milwaukee, WI) for 48 h prior to counting. Control cultures for (1) received SF DMEM only, while (2) received SF DMEM containing 0.02% DMSO. Cell densities were quantified using the Trypan Blue dye exclusion assay. Proliferation assays were repeated 2–3 times with three flasks/treatment/experiment.

**Interference RNA (siRNA)**

Cells seeded at 1.0 × 10^5 cells/25-cm² flask were transfected under SF conditions with 5 or 30 nM siRNA suspended in 8 μL Oligofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Twenty-four hours later, the cultures were washed twice with Hank’s balanced salt solution (HBSS, Invitrogen), retransfected for an additional 24 h with 5 or 30 nM siRNA coupled with 200 ng/mL rhEGF with/without AG1478/SB431542 (200 nM), and then assayed for cell motility. Control cultures were exposed to rhEGF alone, or were retained in SF DMEM. siRNAs treatments targeted mRNA transcribed from STAT3 (Hs_STAT3_1HP; Si00048363; Qiagen), ITGB1 (Hs_ITGB1_1HP; Si00003463; Qiagen), MMP1 (104015; Applied Biosystems), MMP3 (105330; Applied Biosystems), and EGFR (Hs_EGFR_1HP; Si00074032; Qiagen). Gene silencing was monitored by real-time RT-PCR as recommended by Qiagen.

**Statistical Analysis**

Statistical analyses were performed using the Microsoft® Office Excel 2003 statistical analysis tools option. Real-time RT-PCR cycle raw scores were normalized by subtracting the sample’s GAPDH cycle number from each gene’s cycle number. The resulting values were tested for randomness and normality (http://home.ubalt.edu/ntsbarsh/stat-data/Javatstat.htm) prior to applying the parametric analysis. The interrelationships among mRNA concentrations were analyzed using Pearson’s correlation coefficients employing the Bonferroni adjustment for multiple independent significance tests (http://www.quantitativeskills.com/sisa/calculations/bonfer.htm), or by multiple regression using the least squares method. Scatter plots were generated to ensure correlations were not unduly influenced by outliers. Two-tailed t-tests screened for differences in mRNA concentrations. ANOVA was used to test for differences in cell proliferation. The results are expressed throughout as the mean ± standard error of the mean (SEM).

**RESULTS**

**EGF/TGFα/EGFR mRNA Content in Desmoid Tumors**

All 18 desmoid tumors tested positive for EGF, TGFβ, and EGFR mRNA based on real-time RT-PCR. TGFβ transcript concentrations were higher than EGF levels in 13 of the 18 tumors (median fold-difference = 3.6-fold higher; range = 1-fold–580-fold), while in four tumors, EGF content was higher than TGFβ (median = 6.0-fold higher; range = 1.2-fold–50-fold); one tumor contained equivalent concentrations of EGF and TGFβ mRNA. EGF and TGFβ transcript concentrations did not differ significantly within the 18-tumor cohort (t = 0.79; p = 0.43; 19 df), nor did the mRNA content of the two growth factors correlate (r = 0.22; p = 0.39). EGFR mRNA, on the other hand, significantly exceeded the transcript concentrations of both growth factors in the tumors, averaging 70-fold higher than TGFβ content (t = 17.7; p < 0.001; 19 df), and 155-fold higher than EGF (t = 8.6; p < 0.001; 19 df). Continuous in vitro cultivation for one month (i.e., through cell passage #1) following tumor dissociation did not alter the mRNA concentrations of EGF and EGFR from the levels apparent in the parent tumor (SS07-092), but did reduce TGFβ transcript content 50-fold.

Multiple regression analysis using EGF and TGFβ as predictor variables, and EGFR as the response variable,
indicated the variance in EGFR mRNA content within the 18 tumors was best predicted by TGFα transcript content ($p = 0.0018$, coefficient $= 2.2$, $t = 3.8$). EGF on the other hand showed no significant association with EGFR transcript concentration in tumors ($p = 0.26$, coefficient $= 0.18$, $t = 1.17$). TGFα transcript content was reduced substantially when cells were maintained in vitro in complete medium, therefore we did not perform a comparable multiple regression analysis utilizing control culture mRNA concentrations.

**Proliferation Assays**

The cell densities of desmoid cultures stimulated with 100–200 ng/mL rhEGF for 24 h, or exposed to 400 nM AG1478 for 48 h, did not differ from untreated controls ($F = 1.45; p = 0.25$, ANOVA). The average cell density of rhEGF-induced cultures equaled 84% (SEM = 7.2) of untreated control cell densities, while AG1478 treated cultures averaged 101% (SEM = 5.6) of control cultures.

**rhEGF Enhanced Desmoid Cell Motility**

There was a direct dose-dependent relationship between rhEGF stimulation and desmoid cell motility (Fig. 1). Desmoid cell cultures treated for 24 h with rhEGF at concentrations ranging from 5 to 100 ng/mL showed a twofold–threefold enhancement in cell motility compared to untreated control cultures. rhTGFα, in contrast, was less effective at inducing cell migration.

**AG1478 Partially Blocks rhEGF-Induced Cell Motility**

The extent of rhEGF-induced cell migration could be diminished, but not reduced to untreated control levels, by inhibiting the EGFR with AG1478 (Fig. 2). Treatment of cell cultures with 200 nM AG1478, 24 h prior to and during growth factor stimulation, reduced the level of rhEGF-induced cell motility by 17%, compared to rhEGF stimulated cultures (Fig. 2). Doubling the AG1478 dose to 400 nM reduced cell motility an additional 9%. When the EGFR and TGFβ1 receptors were inhibited simultaneously (both inhibitors at 200 nM), rhEGF-induced cell migration was reduced an additional 12% ($t = 3.32; p = 0.006; 12$ df) beyond the level generated by 200 nM AG1478 alone (Fig. 3), and 5% beyond the level produced by 400 nM AG1478 (see Fig. 2). Treatment of cell cultures with 5 nM siEGFR, prior to and during 200 nM AG1478/SB431542 exposure, did not augment the reduction in cell motility induced by 200 nM AG1478/SB431542 (Fig. 3).

**Interference RNA (siRNA) against Motility/Invasion Genes**

Four genes associated with cell motility/invasiveness (MMP1, MMP3, STAT1, and ITGB1) were evaluated using RNA interference in order to define their respective contributions to rhEGF-induced desmoid cell migration. Across the board, 5 nM siRNA proved more effective at reducing rhEGF-induced cell motility than did 30 nM siRNA (Fig. 4), and 5 nM siITGB1 was the most effective of the four siRNAs employed,
then treated with an exogenous source of TGF and were serum starved for 24 h prior to stimulation, and of serum depravation. Since the cells in our experiments reinitiated in cultures sometime during the reinitiated in cultures sometime during the

in vitro culture in medium containing 15% FBS reduced growth factors and then quantified the their effects on cell motility. We found that rhEGF consistently induced desmoid cell motility in a dose-dependent manner.

EGFR activates actin polymerization and lamellipodial protrusion in cells, and presumably both EGFR and TGFβ serve as appropriate ligands. However, rhTGFβ was less effective at inducing motility in the desmoid cultures. The inability of TGFβ to effectively stimulate cell motility may be due to the presence of FBS in the maintenance culture medium. FBS contains retinol (vitamin A) which has been suggested to inhibit TGFβ transcription. We found that 1 month of continuous in vitro culture in medium containing 15% FBS reduced TGFβ mRNA content 50-fold, compared to the parent tumor. On the other hand, TGFβ transcription was reinitiated in cultures sometime during the first 12–18 h of serum deprivation. Since the cells in our experiments were serum starved for 24 h prior to stimulation, and then treated with an exogenous source of TGFβ (also under SF conditions), this combination should have supplied sufficient TGFβ ligand to activate the EGFR, if TGFβ is a mediator of cell motility.

To validate our findings regarding rhEGF stimulation and enhanced cell motility, we inhibited the EGFR with AG1478 and monitored changes in cell migration. AG1478 is a receptor tyrosine kinase inhibitor which preferentially blocks the EGFR kinase without reducing expression of EGFR. The half maximal effective concentration (EC50) of AG1478 is 0.13 ng/mL (~0.4 nM) for proliferating BALB/3T3 cells (Sigma). We found that rhEGF-induced motility could be diminished, but not reduced to control levels, by inhibiting the EGFR with AG1478, even when the drug was applied at molar concentrations (e.g., 400 nM) known to inhibit downstream EGF signaling molecules such as ERK1 and ERK2. Furthermore, pretreatment of cell cultures with siEGFR, followed by combined siEGFR/AG1478/SB431542 treatment, failed to reduce the level of cell motility beyond that induced by the two receptor inhibitors alone.

There are two possible explanations for this outcome: (1) The AG1478 doses applied (200–400 nM over 48 h) were inadequate to mediate full receptor inhibition, or (2) EGF can signal through receptor(s) other than EGFR, if EGFR is compromised (e.g., through mutation). Treatment of PC-3M human prostrate cancer cells with 100 nM AG1478 completely abolished EGF-induced Nav1.7 transcription, suggesting that even at nanomolar concentrations, AG1478 effectively blocks EGF signaling molecules associated with prostrate cancer invasiveness. The fact that low doses of AG1478 (e.g., 100 nM) have proven to be effective in attenuating EGF signaling in other in vitro models, coupled with the fact that RNA interference (siEGFR) combined with AG1478/SB431542 failed to reduce cell motility in our experiments beyond the level resulting from AG1478/SB431542 treatment alone, suggests EGF can signal through receptors other than EGFR. This possibility may explain why EGFR expression level does not define which tumors respond to anti-EGFR therapy, and why only a minority of all EGFR-positive tumors respond to therapy. We found that the simultaneous inhibition of both EGFR and TGFβ receptors was more effective at reducing cell motility than was inhibiting either receptor alone. This raises the possibility that EGF and TGFβ (or their respective receptors) communicate (i.e., “cross-talk”), and that EGF signaling might be accomplished via the TGFβ1/receptor pathway if the traditional EGF/EGFR signaling pathway is compromised.

In conclusion, MMP1, MMP3, STAT3, and ITGB1 respond to EGF stimulation and all are involved in cell motility. By employing RNA interference against the four genes, we identified ITGB1 as the gene most responsible for mediating EGF-induced cell motility in the desmoid cell cultures. ITGB1 is a multifunctional protein involved in cell-matrix adhesion, cell signaling, and receptor-mediated activity. Since anti-EGFR therapy appears relatively ineffective, targeting ITGB1 or other genes involved directly in the physical process of cell motility might enhance the response rate, especially when combined with growth factor receptor therapy.

ACKNOWLEDGMENTS

This study was supported by research grants from the Desmoid Tumor Research Foundation and the Huntsman Desmoid Tumor Research Foundation and the Huntsman

Figure 4. RNA interference for 48 h at 5 nM proved more effective at reducing rhEGF-induced cell motility than did 30 nM siRNA, and 5 nM siITGB1 was the most effective of the four siRNAs employed, while siMMP3 proved least effective. RNA interference typically induced a 2-fold–14-fold reduction in mRNA content in siRNA-treated cultures compared to untreated control cultures when assayed 24 h posttreatment.

DISCUSSION

The association between desmoid growth/invasiveness and the upregulation of growth factors during pregnancy or following trauma suggests a cause-and-effect relationship between elevated and/or prolonged growth factor stimulation and desmoid activity. Hypothesis by exposing desmoid cell cultures in vitro to vitamin A which has been suggested to inhibit TGF

Figure 4. RNA interference for 48 h at 5 nM proved more effective at reducing rhEGF-induced cell motility than did 30 nM siRNA, and 5 nM siITGB1 was the most effective of the four siRNAs employed, while siMMP3 proved least effective. Bars show one standard error (SEM).
REFERENCES