Fas Death Pathway in Sarcomas Correlates with Epidermal Growth Factor Transcription

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Abstract Modulation of apoptosis may influence sarcoma pathogenesis and/or aggressiveness. The Fas death pathway, mediated by FasL or TGF β , is one of two apoptotic pathways. Recent studies report that EGF can modulate TGF β and/or FasL expression/activity; thus, EGF has the potential to influence activation of the Fas pathway. EGF is not always detectable in mesenchymal tumors; therefore, we hypothesized EGF would define which Fas ligand predominates. We assayed 57 surgically removed human sarcomas for 10 genes involved in the Fas pathway. Skeletal muscle biopsies from eight patients served as controls. Sample transcripts were detected by real-time RT-PCR. We attempted to identify relevant predictor variables. The 57 sarcomas were segregated into two categories defined by EGF mRNA content: (1) 23 tumors with EGF concentrations that approximated muscle EGF transcript levels (high-EGF tumors); and (2) 34 tumors that either lacked EGF mRNA, or whose mRNA levels were very low

and frequently undetected by PCR (low-EGF tumors). TGF β 1 expression best predicted Fas transcript concentrations in the 34 low-EGF sarcomas, while FasL predicted Fas mRNA levels in the remaining 23 high-EGF sarcomas. The results suggest ligand activity in the Fas death pathway correlates with EGF transcription in sarcomas.

Introduction

Chemotherapy alone or in combination with radiotherapy is frequently employed against a variety of mesenchymal tumors to induce tumor shrinkage, thus facilitating surgical resection [5, 7, 8, 29, 36]. Most chemotherapeutic agents accomplish this task by inducing apoptosis [10, 32] through the induction of a death receptor [35] or by activation of the endogenous mitochondrial death pathway [12, 23]. Both apoptotic pathways lead individually or collectively [22] to the release of cytochrome c, the activation of initiator and effector caspases [3, 16, 26, 27, 28, 38] and, ultimately, to cell shrinkage, pyknosis, and karyorrhexis [21, 34].

In an earlier study, we reported oncogene coexpression in primary sarcomas correlated with epidermal growth factor (EGF) transcription [19]. Transcript levels were quantified by real-time polymerase chain reaction (RT-PCR) across a pooled cohort of 42 primary sarcomas, representing 12 recognized histotypes. Two genes were considered to be coexpressed if their respective messenger RNA (mRNA) concentrations correlated significantly. Correlations among the 15 oncogenes predominated in sarcomas characterized as EGF-negative (the sarcomas lacked EGF mRNA, or EGF mRNA was detected inconsistently by RT-PCR due to very low transcript concentrations) while, on the other hand, we observed a

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lack of correlation for those same genes when assayed in EGF-positive sarcomas (mRNA was detected in 100% of RT-PCR submissions). Four of these 15 genes (Fas, CTGF, Bcl-2, BAX) are intimately involved in the implementation or inhibition of apoptosis. A strong correlation between Fas (tumor necrosis factor receptor superfamily, member 6; CD95/APO-1) and connective tissue growth factor (CTGF) was defined for the EGF-negative sarcomas, which served as the impetus for the current study. Fas is a cell surface receptor which, upon activation by a ligand, induces the Fas-associated death pathway (FADD) [15]. CTGF may be involved in the induction or inhibition of the FADD pathway by way of its inactivation of the Smad inhibitor SMAD7 [43], or through its general association with TGF β signaling [9, 17].

The dichotomy in Fas:CTGF coexpression apparent in EGF-negative and EGF-positive sarcomas [19] suggested to us that EGF may be involved in the FADD apoptotic pathway. Recent studies reported EGF can inhibit or stimulate Fas-induced apoptosis [14, 20, 25], and the EGF receptor (EGFR) is frequently upregulated in response to chemotherapy [11, 42]. EGF can also modulate TGF β and/or Fas/FasL expression and activity [14, 40, 45]. Hypothetically, EGF could interact with the FADD pathway by modulating Fas ligand activity.

We therefore hypothesized: (1) the two sarcoma categories will differ as to which Fas ligand best describes the variance in Fas, CASP8 and CASP10 mRNA content, and (2), we will be able to identify within the entire 57 sarcoma cohort a tumor EGF mRNA concentration where ligand predominance shifts from one ligand to the other.

Materials and Methods

We quantified in 57 primary sarcomas the mRNA content of 10 genes reportedly involved in the FADD pathway. We segregated the 57 tumors by EGF mRNA concentration into high-EGF and low-EGF tumor categories, and then evaluated the two tumor categories individually using a multiple regression model. For the analysis, Fas, CASP8, and CASP10 served individually as dependent variables, while FasL, TGF β , and EGF served as the predictor variables. Our 57 sarcomas consisted of 52 tumors representing 11 histotypes, plus an additional five tumors reported by the pathologist as “sarcoma of unspecified histotype” (Table 1). For comparative purposes, tumor EGF transcript concentrations were quantified and then compared against the average EGF mRNA content of eight skeletal muscle samples [19].

We selected 10 genes involved in the Fas death pathway for evaluation. In addition to EGF, Fas, and the two Fas ligands, FasL and TGF β 1, we also assayed sarcomas for

Table 1. Primary sarcoma tumor histotypes included within this study

Sarcoma histotype	High-EGF	Low-EGF
Chondrosarcoma	3	3
Ewing's family of tumors	3	1
Fibrosarcoma	1	0
UpS	3	3
Hemangiopericytoma	3	0
Leiomyosarcoma	4	2
Liposarcoma	0	3
Osteosarcoma	1	5
MPNST	4	0
Rhabdomyosarcoma	0	1
Synovial	0	12
Undefined sarcoma	1	4

UpS = undifferentiated high-grade pleomorphic sarcoma; MPNST = malignant peripheral nerve sheath tumor.

CASP8, CASP10, and CFLAR. CASP8 and CASP10 are two initiator proteases responsible for the activation of downstream effector caspases, while CFLAR inhibits Fas-induced cell death by blocking recruitment and processing of CASP8 at the death-inducing signaling complex (DISC). CFLAR also interacts with CASP10. SMAD3 plus the SMAD3-inhibitor SMAD7 were included because they are transcriptional modulators activated by TGF β 1 and, in the case of SMAD7, by EGF, and because SMAD3 interacts directly with Fas. We also included CTGF, as this gene may also be involved in smad-mediated TGF β -induced cell death [18, 39]. Real-time RT-PCR was performed using the ABI PRISM® 7900 HT sequence detection system with primer sets (TaqMan® gene expression assays) supplied by Applied Biosystems (Foster City, CA).

Sarcomas were segregated by EGF mRNA content into “high-EGF” or “low-EGF” categories based on the frequency of detection of EGF mRNA by real-time RT-PCR. Tumors were classified as high-EGF if EGF transcripts were detected consistently by real-time RT-PCR. The 23 sarcomas placed into the high-EGF category represented 16 of the 27 EGF-positive sarcomas reported previously [19], plus an additional seven sarcomas processed specifically for this study. “Low-EGF” sarcomas, however, lacked EGF mRNA, or EGF transcripts were detected inconsistently. The low-EGF sarcoma category consisted of 15 EGF-negative sarcomas reported previously [19], plus an additional 19 tumors.

For the statistical analysis, we normalized the real time RT-PCR data by dividing each gene's cycle number by the tumor's GAPDH cycle number. The ABI PRISM® 7900 HT sequence detection system reports an mRNA concentration as “the cycle when amplification of the target is first detected; the higher the starting copy number of the nucleic

acid target, the sooner a significant increase in fluorescence (ie, the smaller the cycle number) is observed.” Cycle values for our sarcoma cohort ranged from 18 to 39. Six of the 57 sarcomas lacked detectable levels of EGF mRNA and did not generate a cycle number. In order to accommodate the analysis, we arbitrarily assigned an EGF cycle value of 40 to those six tumors. Forty represented one cycle greater than the highest cycle value produced by the lowest detectable level of EGF mRNA (ie, cycle 39). The two sarcoma categories were then analyzed separately by multiple regression using the least squares method (Microsoft Office Excel statistical analysis tools), with TGFβ1, FasL, and EGF as predictor variables, and Fas, CASP8, or CASP10 as the response variable. To identify the transition point where ligand predominance shifted from one ligand to the other, we combined the high- and low-EGF sarcoma categories into a single 57-tumor cohort, and then sorted the 57 tumors by descending EGF mRNA content. We then performed eight successive, partially overlapping, 20-tumor multiple regressions that collectively incorporated 55 of the 57 tumors. The first regression evaluated tumors one through 20 (all 20 were high-EGF tumors), the second regression evaluated tumors six through 26 (18 high-EGF and two low-EGF), and so forth. The eighth regression consisted of 12 tumors with very low concentrations of EGF mRNA, plus four of the six tumors lacking EGF.

Tissue acquisition, mRNA purification, and real-time RT-PCR follow Joyner et al. [19]. We used Pearson correlation coefficients (r) to identify gene associations ($p \leq 0.005$, employing the Bonferroni adjustment for multiple independent significance tests) [2]. We specifically tested for potential associations among the 10 FADD-related genes using correlation coefficients. Scatter plots were generated to ensure correlations were not unduly influenced by outliers. We also sorted the entire cohort of 57 sarcomas by descending EGF mRNA content and then used eight, successive and partially overlapping multiple regressions involving 20 tumors per regression to search for a tumor EGF concentration where TGFβ replaced FasL as the best predictor of Fas content.

Results

The variance in Fas mRNA content in the 23 high-EGF sarcomas was best predicted by FasL mRNA concentrations, while TGFβ1 predicted Fas transcript levels in the 34 low-EGF tumors (Table 2). The apparent dichotomy in ligand activity can be illustrated with the use of scatter plots (Figs. 1, 2). Fas concentrations in high-EGF tumors were plotted as a function of FasL (Fig. 1A), or TGFβ1 (Fig. 1B). The respective coefficients of determination (R^2)

Table 2. FasL predicts Fas mRNA content in high-EGF sarcomas, while TGFβ1 predicts Fas mRNA content in low-EGF sarcomas

Tumor EGF mRNA content	Gene	Partial coefficient	t Statistic	Probability
High-EGF	<i>TGFβ1</i>	0.17	0.51	0.62
	<i>FasL</i>	0.28	2.73	0.01*
Low-EGF	<i>TGFβ1</i>	1.02	5.76	5.3E−06*
	<i>FasL</i>	0.04	0.46	0.65

*Significant probability values.

Fig. 1A–B Tumor necrosis factor receptor superfamily, member 6 (Fas; ordinate) mRNA content in high-EGF sarcomas correlates with (A) tumor necrosis factor ligand superfamily, member 6 (FasL; abscissa; $p = 0.002$, $N = 23$), but not with (B) transforming growth factor, beta-1 (TGFβ1; abscissa; $p = 0.80$, $N = 23$). Graph axes represent real-time RT-PCR estimates of FAS, FasL, and TGFβ1 mRNA concentrations adjusted by tumor GAPDH. Curves are linear trendlines.

clearly reflect a preference for FasL in high-EGF sarcomas. On the other hand, in low-EGF sarcomas, TGFβ1 (Fig. 2B) corresponds much more closely with Fas than does FasL (Fig. 2A). TGFβ1 also served as the best predictor of caspase mRNA content in low-EGF tumors, while FasL predicted CASP8 mRNA in high-EGF tumors (Table 3).

The EGF mRNA concentrations of the 57 sarcomas ranged from a seven-fold enhancement over skeletal muscle EGF content to a 977-fold reduction in EGF mRNA content

Fig. 2A–B Tumor necrosis factor receptor superfamily, member 6 (Fas; ordinate) mRNA content in low-EGF sarcomas does not correlate with (A) tumor necrosis factor ligand superfamily, member 6 (FasL; abscissa; $p = 0.06$, $N = 34$), but does with (B) transforming growth factor, beta-1 (TGF β 1; abscissa; $p = 0.00001$, $N = 34$). Graph axes represent real time RT-PCR estimates of FAS, FasL and TGF β 1 mRNA concentrations adjusted by tumor GAPDH. Curves are linear trendlines.

Table 3. Caspase-8 (CASP8) and caspase-10 (CASP10) mRNA content is also defined by sarcoma mRNA concentration

Tumor EGF mRNA content	Response gene	Predictor gene	t Statistic	Probability
High-EGF	CASP8	TGF β 1	0.96	0.35
		FasL	2.5	0.02*
	CASP10	TGF β 1	0.79	0.44
		FasL	1.2	0.26
Low-EGF	CASP8	TGF β 1	2.1	0.04*
		FasL	-0.05	0.96
	CASP10	TGF β 1	3.3	0.002*
		FasL	2.1	0.05*

*Significant probability values.

relative to muscle, and six of the 34 sarcomas lacked detectable levels of EGF mRNA altogether (Table 4). A transition from FasL to TGF β 1 became apparent when the average EGF mRNA concentration was approximately 12-fold below the EGF mRNA content of skeletal muscle

Table 4. Range of tumor EGF mRNA content as a fold-change over skeletal muscle mRNA content

Tumor EGF mRNA content	Number of tumors	Tumor EGF mRNA content as a fold-change over skeletal muscle mRNA content
High-EGF	23	7.3-fold > muscle to 17-fold < muscle
Low-EGF	34	21-fold to 977-fold < muscle (six tumors showed no detectable EGF mRNA.)

(Table 5). FasL predominated in tumors with higher EGF concentrations, while TGF β 1 served as the only predictor of Fas content in sarcomas with lower EGF concentrations. We found few genes correlated in the high-EGF sarcoma category. Only five of 45 potential gene correlations were detected in high-EGF tumors, which included FasL with Fas ($r = 0.57$; $p = 0.004$), but not TGF β 1 with Fas ($r = 0.29$; $p = 0.18$). In comparison, 24 of 45 potential correlations (53%) were detected in low-EGF sarcomas. A majority of the mRNA correlations represented in low-EGF sarcomas but not apparent in high-EGF tumors involved TGF β 1, Fas, SMAD3 and SMAD7. Included within the low-EGF category was a correlation between TGF β 1 and Fas ($r = 0.809$; $p = 6.9\text{E-}09$). Correlations between CASP8 and CASP10 or between CFLAR and either caspase occurred irrespective of tumor EGF status.

Discussion

We previously reported EGF mRNA is widely expressed in skeletal muscle and in mesenchymal tumors, but not all mesenchymal tumors are EGF positive [19]; EGF mRNA presence appears independent of tumor histotype, and does not correlate with benign versus malignant status; and judging from the contrasting pattern of oncogene synchronization between EGF-positive and EGF-negative tumors, EGF either (1) actively regulates gene synchronization in mesenchymal tumors, or (2) serves as an indicator of tumor gene synchrony and, therefore, may not be directly responsible for the coordinated expression of other gene pairs. Four of the genes monitored in that study are involved in the implementation or inhibition of apoptosis. Since the mRNA content of the four genes correlated with EGF content, we asked whether there might be an association between EGF expression and apoptosis in sarcomas. We limited our search to the Fas-associated death pathway (FADD) because two of the four genes, Fas and CTGF, are directly involved in this pathway.

We made several decisions during the design of our study that potentially could have biased our results. We pooled 52 sarcomas representing 11 histotypes plus an

Table 5. A shift in Fas ligand functionality from FasL to TGFβ1 occurs when tumor EGF mRNA concentrations are 2-to-30-fold lower than skeletal muscle EGF levels

Regression	High-EGF to low-EGF tumor ratio	TGFβ1 probability	FasL probability	Mean fold-change over muscle EGF mRNA (range)
1st	20:0	0.98	0.01	−1.5 (7.3 to −9)
2nd	18:2	0.38	0.04	−6.1 (1.6 to −17)
3rd	13:7	0.10	0.33	−12.0 (−2 to −30)
4th	8:12	0.002	0.53	−19.3 (−4 to −38)
5th	3:17	0.0003	0.73	−31.6 (−10 to −76)
6th	0:20	0.0002	0.76	−54.5 (−18 to −139)
7th	0:20	0.00005	0.97	−116.7 (−30 to −374)
8th	0:20	0.00006	0.78	−280.1 (−38 to −977)

additional five unclassified sarcomas into a single 57-tumor cohort. We believed an analysis of this multihistotype cohort was appropriate because previous studies demonstrate including multiple gene ontology (GO) categories or multiple species in an analysis of gene expression reduces the noise and improves function prediction [1, 41, 44]. Furthermore, our data reflect the expression profiles of established tumors, so we assumed there is a commonality in FADD function that transcends sarcoma cell-of-origin.

A second decision involved our segregation of the 57 sarcomas into high-EGF and low-EGF categories, based entirely on the frequency of detection of tumor EGF mRNA by real-time RT-PCR. This decision appears to have been fortuitous because our results show a correlation between EGF expression in sarcomas and Fas ligand functionality. If our eight “independent,” 20-tumor multiple regressions provided a reasonably accurate prediction of when the shift in Fas ligand functionality takes place, the transition occurs when tumor EGF transcript content is approximately 12 times less than the average EGF transcript content of skeletal muscle. Unfortunately, we cannot provide an estimate of EGF transcript copy number corresponding to that 12-fold reduction, because we lack a suitable reference plasmid of known size and known copy number, which would have been used to define the constituent copy number of EGF transcripts.

Lastly, we utilized a parametric regression model for our analysis. We understand that the reliability of the predictions derived from this statistical model is sample-size dependent, and our division of the original 57-tumor cohort into two EGF-related categories reduced a limited sample size even further. However, the pattern reflecting FasL functionality in high-EGF tumors compared to TGFβ1 in low-EGF tumors remained reasonably consistent through to CASP8 and CASP10, two genes that are downstream of and activated by Fas. We also observed what appeared to be a transition point where FasL was replaced by TGFβ1 as the ligand that best explained the variance in Fas mRNA

content [13]. In spite of the limited sample sizes, taken together, these findings suggest that Fas ligand functionality may be mediated directly or indirectly by EGF.

We suggest there is a plausible explanation for the apparent shift in Fas ligand functionality that takes into account tumor EGF content. This scenario involves EGF activation of phosphatidylinositol 3'-kinase (PI 3-kinase) and the subsequent phosphorylation of Akt/PKB serine/threonine kinase. The PI 3-kinase pathway is activated following EGF stimulation [24,30, 33]. PI 3-kinase in turn phosphorylates phosphatidylinositides on the 3' position to produce phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂ and 3,4-triphosphate (PtdIns(3,4)P₃ [46]. PtdIns(3,4)P₂ preferentially activates the Akt/PKB serine/threonine kinase signaling pathway [4], which inactivates SMAD3 by sequestering unphosphorylated SMAD3 in the cytoplasm, thus preventing SMAD3 phosphorylation and nuclear translocation [6, 37]. Sequestration of unphosphorylated SMAD3 inhibits TGFβ1-induced, SMAD3-mediated transcription and apoptosis. The sarcomas we utilized represent various stages of this sequential process. We argue FasL is operational, perhaps by default, when the EGF content of sarcomas is high. However, as the concentration of EGF diminishes, TGFβ1-induced apoptosis is no longer inhibited by the sequestration of SMAD3; therefore, TGFβ1 becomes the dominant stimulator of the FADD pathway in sarcomas [31].

Our study also suggests coexpression of FADD-related genes predominates in low-EGF sarcomas. This conclusion is consistent with our earlier findings regarding the coexpression of oncogenes and tumor suppressor genes in sarcomas [19].

Fas ligand functionality correlated with, and may be modulated in sarcomas by EGF, possibly via the PI 3-kinase/Akt pathway. Targeting of EGF, its receptor, or components within the PI 3-K/Akt pathway may influence the clinical behavior of sarcomas. FasL, applied at subtoxic doses, sensitizes soft tissue sarcomas to therapeutic drugs

[26]. In a similar manner, manipulation of Fas ligand functionality may also enhance sarcoma susceptibility to drug-induced apoptosis. For example, treating sarcomas with recombinant human EGF may favor FasL functionality over TGF β 1, while simultaneously reducing or inhibiting SMAD3-mediated, TGF β 1-induced cell survival through the cytoplasmic sequestration of unphosphorylated SMAD3.

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