Footprinting of Individual Tumors and Their Variants by Constitutive Cytokine Expression Patterns

Lisa A. Pekarek,1 Ralph R. Weichselbaum, Michael A. Beckett, James Nachman, and Hans Schreiber

Abstract

We have examined the cytokine mRNA expression profile of six different human cell lines derived from Ewing sarcomas by using polymerase chain reaction and found each to constitutively express a characteristic pattern. Furthermore, each cell line differed in the levels of secreted cytokines. We also analyzed the expression of several cytokines in murine UV-induced sarcomas and their heritably stable progressive variants. Each murine tumor also constitutively expressed a large number of cytokines, and in some cases, the more malignant variants differed from their parental tumors. These results demonstrate that tumors of the same type, and even in the same lineage, can have distinct cytokine expression and/or secretion profiles. Some cytokines may stimulate tumor growth while others may have antitumor effects. Cytokine therapy may be tailored depending upon the cytokine profile of the individual malignancy.

Introduction

Cytokines are effective in altering the growth of malignant cells in vivo. This has been demonstrated in numerous experimental systems by transfecting cytokine genes into tumor cells (for a review, see Ref. 1). For example, highly malignant cells transfected to produce TNFα may be markedly growth inhibited for a prolonged period of time even in the absence of T-cell immunity (2). Also, IL-4 and G-CSF are reported to exert strong tumor suppressing effects which appear to be mediated primarily through the induction of non-T-effector cells (3, 4). In addition, several cytokines such as INFγ, IL-2, IL-4, IL-7, and TNFα when secreted by transfected tumor cells could inhibit tumor growth, at least in part by enhancing T-cell-mediated tumor immunity (for a review, see Ref. 1). Cytokines may also promote tumor growth under certain conditions. For example, TGFβ, IL-3, IL-5, IL-6, IL-9, and GM-CSF may promote tumor growth by autocrine stimulation when released by certain transfected cells (for a review, see Ref. 1). The effect of a specific cytokine may depend upon both the particular tumor cell line studied and the influences of other cytokines already produced by those cell lines. Cytokines may also alter the malignant behavior by acting on the tumor cells directly, or by involving host components.

Tumor cells of hematopoietic origin in particular have been reported to produce cytokines constitutively (5) and several of these have been suspected of acting as autocrine growth regulators. Little is known about the production of cytokines by nonhematopoietic malignancies even though solid tumors represent the majority of human cancers. In this report, we have analyzed cell lines derived from two types of nonhematopoietic malignancies: (a) human Ewing sarcomas; and (b) murine UV-induced sarcomas. We report constitutive expression of mRNA for numerous cytokines as well as secretion of several of these cytokines at levels comparable to those observed in cytokine gene-transfected tumor cells. We found repeatable characteristic differences in cytokine profiles, not only between individual tumors, but also between the parental tumors and their variant derivatives.

Materials and Methods

Tumor Cell Lines. Methods of establishment of human sarcomas and maintenance of cell cultures have been described (6). Each of the human sarcomas used were diagnosed as Ewing sarcomas at the time of biopsy. STSAR 11 and 33 were later shown to have the t(11;22)(q24;q12) translocation breakpoint characteristic for Ewing sarcoma (7). The other four (STSAR 71, 72, 264, and 318) had chromosomal aberrations in some cells and a transformed morphology. Each is at approximately the 25th passage. STSAR 71 and 72 were obtained from separate biopsy samples from the same patient. The murine UV-induced RE tumors are rejected when tumor fragments are transplanted into syngeneic mice, while heritable PRO variants, derived from the RE tumors, lethally progress upon transplantation. Derivation and culture of UV-induced tumors in C3H/HeN mice and of the PRO tumor variants have been described (8, 9).

Preparation of RNA and cDNA. Five × 10⁶ to 1 × 10⁹ tissue culture cells were lysed in RNAzol B (Tel-Test, Inc., Friendswood, TX) and total RNA was purified as prescribed by the manufacturer. Two μg of total RNA were reverse-transcribed by the addition of 500 pmol random hexamers (GIBCO/BRL, Gaithersburg, MD), 500 μm deoxynucleotides (Boehringer Mannheim, Indianapolis, IN), 1 × Moloney murine leukemia virus reverse transcriptase buffer, and 400 units Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in a 40-μl volume. Reactions were incubated at 37°C for 60 min and then heated inactivated at 90°C for 10 min.

PCR Conditions. Human cytokine primers have been published (10) except for:

GM-CSF sense primer, 5'-CATGTAATGCCCATCCGGAG-3';
GM-CSF antisense primer, 5'-AGTGTCTCTTTTGAATTGCTGGCCATCATGG-3';
G-CSF sense primer, 5'-AGTGCACTCTGGACAGTGCAAGGCA-3';
and
G-CSF antisense primer, 5'-CTGCCAGATGGTTGCGACAGGCA-3'.

Murine cytokine primers have been published (11) except for:

IL-1β sense primer, 5'-CTGGAAGCACTATGGCAACT-3';
IL-1β antisense primer, 5'-GGATGCTCTCATTCAGGACAG; TGFβ sense primer, 5'-TATAGCAAGCTTTCGCGCT-3';
TGFβ antisense primer, 5'-TCTCCAAATGTCAATGACCT-3';
GM-CSF sense primer, 5'-CATGTGTCGAGACCGCCTGAGAT-3';
GM-CSF antisense primer, 5'-GACATCAAGGAGGATCATGAGC-3';
G-CSF sense primer, 5'-GTTGGTGCGACACCCTAAGGCTG-3';
and
G-CSF antisense primer, 5'-GGCAATGGTGCCACATCCAGTGA-3'.

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2 To whom requests for reprints should be addressed, Department of Pathology, The University of Chicago, 5841 South Maryland Ave., MC1089, Chicago, IL 60637.
3 The abbreviations used are: TNFα, tumor necrosis factor α; IL, interleukin; PCR, polymerase chain reaction; GM-CSF, granulocyte macrophage-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; TGFβ, transforming growth factor β; LT, lymphotoxin; INFγ, interferon; RE, regressor; PRO, progressor; DNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; CTL, cytotoxic T-lymphocyte.
CONSTITUTIVE CYTOKINE EXPRESSION PATTERNS

PCR amplifications were performed using 250 ng of each primer, 3–6% of the cDNA reaction, 250 μM deoxynucleotides, 0.5 units Taq polymerase, and 1 × buffer (Boehringer Mannheim) in a total volume of 100 μl. PCR components were routinely checked for contamination by amplifying 60 cycles in the absence of a template. PCR reactions were subjected to denaturation at 94°C for 1 min, annealing at 72°C for 1.5 min, and extension at 55°C for 2 min for 40–60 cycles using a Perkin Elmer DNA Thermal Cycler 480.

**Cytokine ELISA.** Assays for human cytokines IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, G-CSF, and GM-CSF were performed using commercial kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s specifications.

**Bioassays.** Bioassays for INFγ, TNFα/LT, IL-2, IL-4, and IL-6 were performed as previously described with a few modifications. Briefly, murine INFγ concentrations were determined using an ELISA and compared to a known standard (12). IL-2 activity was assessed using CTLL cells (12). IL-6 activity was assessed using the factor-dependent cell line B9 (13). TNFα/LT was assayed on TNF-sensitive RE3.5 cells (2). Responses of CTLL, B9, and RE3.5 cells were determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (14) and the titer was compared to standard curves using known concentrations of purified cytokines. TGFβ bioactivity was quantified by its ability to inhibit cDNA synthesis in IL-6 treated cells (15) with slight modifications. Cultured supernatant were activated by the addition of 0.1 M HCl for 30 min and then neutralized with 0.72 M NaOH plus 0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. G-CSF was quantified by its ability to stimulate colony formation of murine bone marrow cells as described (16). The level of detection for the cytokines were as follows: IL-2, ≥13 pg/ml; IL-4, ≥13 pg/ml; IL-6, ≥0.13 units/ml; TNFα/LT, ≥13 pg/ml; INFγ, ≥2 units/ml; TGFβ, ≥1 ng/ml; and G-CSF, ≥2 units/ml. Culture supernatants for Ewing sarcoma cells were generated by incubating 5 × 10⁶ cells for 48 h in 6 ml of serum-free minimal essential medium.Murine supernatants were from 4 × 10⁶ cells in 5 ml minimal essential medium supplemented with 0.1% fetal calf serum.

**Results**

**Individual Tumors of the Same Tumor Type Have a Characteristic Pattern of Cytokine mRNA Expression.** A panel of six different cell lines derived from Ewing sarcomas were analyzed for expression of cytokines known to be associated with altered tumorigenicity in experimental systems. RNA was extracted, cDNA was synthesized, and PCR was performed to amplify specific cytokine messages (see “Materials and Methods”). Equivalent amounts of PCR reactions were loaded on agarose gels as seen by comparable β-actin bands (Fig. 1).

Each cell line had a characteristic pattern of cytokine expression (Table 1). These same patterns were obtained from 3 separate RNA extractions performed at different times. All tumor cell lines had high levels of RNA expression for IL-8 and G-CSF, as well as varying levels for TGFβ, GM-CSF, and LT. None of the tumors expressed detectable message for IL-7 or INFγ after 60 cycles of PCR amplification. Anti-CD3-stimulated human peripheral blood lymphocytes, used as positive controls, expressed high levels of both cytokines (data not shown). A human fibroblast line, IMR-90, was analyzed for constitutive cytokine mRNA expression. These cells expressed a large number of different cytokines, and the pattern differed from all of the other tumors (Table 1). Ewing tumors STSAR-71 and 72, derived from separate tumor biopsies from the same patient at different stages of tumor growth, showed different patterns of cytokine message. However, it is difficult to assess for human tumors whether these differences in cytokine expression were found in isolates that differed in aggressiveness of tumor growth. We therefore examined cytokine expression in murine tumors.

**Variants with Increased Malignant Potential Can Differ from the Parental Tumor in Their Pattern of Cytokine mRNA Expression.** We next determined how much cytokine mRNA expression patterns could differ between a tumor and its more malignant variant that developed during tumor progression. We used a series of well characterized regressor tumors along with their progressor variants. The regressor tumors when transplanted into normal mice initially grow but are later rejected while the progressor variants lethally progress (8, 9). Like the Ewing sarcomas, individual murine tumor cell lines had characteristic patterns of cytokine expression (Table 2) even though different cytokine messages were prominent. All murine tumor cell lines expressed mRNA for IL-1α, IL-5, IL-6, INFγ, TGFβ, LT, and G-CSF. No IL-3 could be detected in repeated experiments, although it was detected in normal murine inflammatory cells. For the first two tumors shown in Table 2, the PRO variants differed from their parental tumors in their pattern of cytokine expression. These two variants had been derived in vivo from normal mice that failed to reject the regressor tumor challenge. The patterns reported for 4102-RE and 4102-PRO were from tumor cell clones while those for 6132A-RE and 6132A-PRO were from tumor cell populations. Clones of 6132A-RE and 6132A-PRO have also been analyzed and were found to differ not only from each other, but also from the population from which they were selected. For the three other tumors, the cytokine expression pattern of the parental tumor and its variant was very similar whether clones or populations were analyzed. These three

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**Fig. 1. Comparison of cytokine gene expression in Ewing sarcomas.** Total RNA was extracted from tissue culture cells and was reverse-transcribed into cDNA. cDNA was amplified by PCR using cytokine-specific primers. Sizes of PCR products are in base pairs: β-actin, 548; IL-1α, 516; IL-1β, 810; IL-2, 462; IL-3, 459; IL-6, 639; IL-7, 707; IL-8, 302; TGFβ, 282; LT, 608; TNFα, 702; GM-CSF, 219; and G-CSF, 383. As a control, β-actin was amplified. First lane, 6X174/HaeIII digest marker.

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variants had all been selected in vitro by exposure of the parental tumor to tumor-specific CTL.

**Cytokine Secretion.** The differences in cytokine message patterns among individual human or murine tumors were quite striking. We, therefore, wanted to determine whether any of these cytokines, for which there was detectable message, were actually secreted. Thus, supernatants from Ewing sarcoma cells were analyzed by ELISA or bioassay (Table 3). Assays were performed twice on separate supernatants. Message for IL-1α, IL-1β, IL-2, IL-3, TNFα, and LT could be detected in one or several cell lines. However, none of these cytokines appeared to be secreted, at least not without exogenous stimulation. TNFα was previously found to be secreted by STSAR 33 (17) at 0.17 ±. weak band detectable after amplifying 60 cycles; —¿, no detectable band after 60 cycles of PCR. TGFß. <1 ng/ml. **Twenty % of the PCR reactions were loaded on a 1.8% agarose gel and visualized by ethidium bromide staining. Results of 3 separate RNA extractions. Derived from cloned variants.**

### Table 1 Cytokine gene expression in Ewing sarcomas as detected by PCR

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| Results of 3 separate RNA extractions. | — —, no detectable band after 60 cycles of PCR; + +, intensity of PCR band is comparable to that of ß-actin for that cell line; +, intensity of PCR band is less than that of ß-actin but clearly detectable after 40 cycles of PCR; ±, weak band detectable after amplifying 60 cycles. | — —, weak band detectable after amplifying 60 cycles; —¿, no detectable band after 60 cycles of PCR; + +, intensity of PCR band is comparable to that of ß-actin for that cell line; +, intensity of PCR band is less than that of ß-actin but clearly detectable after 40 cycles of PCR; ±, weak band detectable after amplifying 60 cycles. | Twenty % of the PCR reactions were loaded on a 1.8% agarose gel and visualized by ethidium bromide staining. Results of 3 separate RNA extractions. Derived from cloned variants. **Twenty % of the PCR reactions were loaded on a 1.8% agarose gel and visualized by ethidium bromide staining. Results of 3 separate RNA extractions. Derived from cloned variants.** |

### Table 2 Cytokine gene expression in murine sarcomas as detected by PCR

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### Table 3 Cytokine secretion by Ewing sarcomas (pg/ml)

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| Twenty % of the PCR reactions were loaded on a 1.8% agarose gel and visualized by ethidium bromide staining. Results of 3 separate RNA extractions. Derived from cloned variants. **Twenty % of the PCR reactions were loaded on a 1.8% agarose gel and visualized by ethidium bromide staining. Results of 3 separate RNA extractions. Derived from cloned variants.** |

### Discussion

The data presented here indicate that individual tumor cell lines, derived from the same histological type, can have characteristic profiles of cytokine mRNA expression. These patterns are repeatable from separate RNA preparations made at different times during cell culture of both human Ewing sarcomas and murine UV-induced sarcomas. A recent study compared profiles of cytokine expression in several melanomas and also found different patterns for different tumors (18). We found that each cell line derived from Ewing sarcomas differed in either the levels or types of cytokines expressed and secreted. Although it could not be determined whether the human tumors express these cytokines in vivo, analyses of cytokine mRNA from biopsies of murine tumors are consistent with those seen in cultured cells.5

Our comparative analysis of murine regressor tumors and the progressor variants suggests a functional importance for such differences in cytokine profiles. Both of the progressor variants that had been derived by selection in vivo differed from the parental tumors in their...
pattern of cytokine expression. Both of these variants also differed remarkably from their parental cell lines in their sensitivity to growth factors including TGFβ in vitro, and grew faster in T-cell-incompetent mice. This was not observed for the progressor variants that had been selected for antigen loss by exposure to CTL in culture. Thus, progressor variants that do not or cannot lose a CTL-recognized antigen may use the cytokine environment in order to escape host control.

It is important that many cytokines were secreted by the tumor cells at levels comparable to those produced by cytokine gene transfectants which had effects on tumor growth. These transfection studies were done without analyzing the pattern of cytokine messages already present in the individual tumors. Multiple cytokines, secreted by the same tumor, could enhance or antagonize each other. Also, the same cytokine may affect various tumor cells differently depending on their stage of malignancy. Tumor cells which are originally inhibited by a certain cytokine may develop more malignant variants which are then stimulated by that cytokine. Both TGFβ and IL-6 have been shown to stimulate more aggressive tumor cells while actually inhibiting less aggressive and normal cells (19, 20). While we do not know that tumor growth or progression is influenced by the production of these cytokines, our results obtained with the two in vivo-derived progressor variants are consistent with such a notion.

Further characterization of the in vivo roles of specific cytokines and their interactions may help to predict the clinical course and possible methods of intervention. Blocking those cytokines which may be growth stimulatory or inducing the production of those that may inhibit tumor growth is possible. Hallahan et al. (17), for example, have found secretion of TNFα in one Ewing sarcoma in vitro after ionizing radiation (17). This was one of the tumors in which we found high levels of TNFα message but no constitutive TNFα protein secretion. Our data emphasize that many cytokines are already present in the individual tumors. Multiple cytokines, secreted by the tumor cells, could enhance or antagonize each other. Also, the same cytokine may affect various tumor cells differently depending on their stage of malignancy. Tumor cells which are originally inhibited by a certain cytokine may develop more malignant variants which are then stimulated by that cytokine. Both TGFβ and IL-6 have been shown to stimulate more aggressive tumor cells while actually inhibiting less aggressive and normal cells (19, 20). While we do not know that tumor growth or progression is influenced by the production of these cytokines, our results obtained with the two in vivo-derived progressor variants are consistent with such a notion.

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References

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