Technical Advance

Isolation of Mouse Stromal Cells Associated with a Human Tumor Using Differential Diphtheria Toxin Sensitivity

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Tumor vascularization is accompanied by the migration of stromal cells, including endothelial cells, smooth muscle cells, and fibroblasts, into the tumor. The biological contributions of stromal cells to tumor vascularization have not been well-defined, partly due to the difficulty of culturing stromal cells in the presence of large numbers of fast-growing tumor cells. To address this problem, a strategy was devised to kill tumor cells but not stromal cells. Advantage was taken of the observation that diphtheria toxin (DT) kills human but not rodent cells. Human melanoma (MMAN) tumor cells were injected subcutaneously into nude mice. The tumors were excised, homogenized, and treated with 50 ng/ml DT for 24 hours. Elimination of melanoma cells by DT treatment was demonstrated by lack of detectable levels of microphthalmia, a transcription factor that is a marker for melanoma cells. The murine stromal cells were viable and found to be mostly smooth muscle cells. These cells constituted about 1.5% of the MMAN tumor. RNase protection assays using a specific murine vascular endothelial growth factor probe confirmed the murine origin of the stromal cells. This method allows rapid isolation of stromal cells and should facilitate biochemical and genetic analysis of tumor-stromal interactions. (*Am J Pathol* 1999, 155:723-729)

Stromal cell-epithelial cell interactions are important for the growth, development, and maintenance of many tissues. Some of these interactions are mediated by peptide growth factors and their receptors. For example, keratinocyte growth factor (KGF), produced by stromal cells in the skin, and heparin-binding EGF-like growth factor (HB-EGF), produced by stromal smooth muscle cells in the prostate, stimulate epithelial cell growth and differentiation in skin and prostate, respectively.^{1,2} Stromal cell-derived KGF also promotes wound healing and stimulation of hair growth in the nude mouse *in vivo*.³ Tumors are invested with stromal elements including endothelial cells, pericytes/smooth muscle cells, fibroblasts, and macrophages. The stromal endothelial cells and smooth muscle-derived pericytes form the tumor microvasculature required for subsequent tumor growth.⁴ These host cells migrate into a developing tumor in response to tumor-derived growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF).^{5,6} The general assumption has been that the growth factors that induce tumor angiogenesis and growth are tumor cell-derived. However, the possibility that tumor-associated stromal cells secrete growth factors such as VEGF, fibroblast growth factor (FGF), and PDGF, and thereby support tumor angiogenesis, growth, and/or survival has not been explored fully.

The biological contributions of stromal cells to tumor vascularization and growth have not been addressed adequately, partly because of the difficulty of obtaining populations of cultured stromal cells free of tumor cells. To address this problem, a strategy was used to kill tumor

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cells but not stromal cells. We took advantage of the observation that diphtheria toxin (DT) kills human but not rodent cells. DT is a very potent bacterially derived toxin for mammalian cells.⁷ It is a heterodimer consisting of two functional subunits, a receptor binding subunit that allows for intracellular translocation and a catalytic subunit that ADP ribosylates the elongation factor, EF-2, resulting in inhibition of protein synthesis followed by cell death.^{8,9} Most animals are highly sensitive to the toxic effects of DT, which is lethal at 100 ng/kg body weight.^{10,11} However, mice and rats are resistant to DT and survive at 1000 times the dose for the susceptible species.⁷ The DT receptor (DTR) has been shown to be the transmembrane form of heparin-binding epidermal growth factor like factor (HB-EGF).^{12,13} The DT binding site on HB-EGF is in the EGF-like domain.¹⁴ Alterations of several amino acids in the EGF-like domain reduce substantially the ability of murine HB-EGF/DTR to bind DT when compared to human HB-EGF/DTR.¹⁵

In this report, we describe the isolation of murine host stromal cells from a human melanoma tumor by differential DT killing of the human tumor cells. These cells were mostly smooth muscle cells but included endothelial cells as well. Differential DT sensitivity is a novel and rapid method for the study of cellular host responses to xenografts including tumors and other pathological processes.

Materials and Methods

Materials

Male nude mice were obtained from Massachusetts General Hospital (Boston, MA) and treated with DT between 5 and 8 weeks of age. Dulbecco's modified Eagle's medium (DMEM) containing 1000 mg/l glucose and RPMI 1640 were obtained from Sigma Chemical Company (St. Louis, MO). DT was obtained from Calbiochem (San Diego, CA).

Cell Culture

MMAN melanoma cells¹⁶ were cultured in DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μ g/ml streptomycin. These cells were maintained in an atmosphere of 10% CO₂. Wild-type 32D cells and 32D cells expressing human HB-EGF were cultured as described.¹⁷

Growth of MMAN Tumor Xenografts and DT Treatment in Vivo

Nude male mice were injected subcutaneously in the right flank with 1×10^6 of MMAN melanoma cells. After approximately 4 weeks, the melanoma tumors reached approximately 1 cm³ in volume. The mice were then treated with 1 μ g DT intraperitoneally. All experiments were repeated in triplicate.

DT Treatment of Melanoma Tumors in Vitro

Melanoma tumors were removed from animals under aseptic conditions. The tumors were minced with scissors into 1-mm cubes, which were digested in 15 ml of phosphate buffered saline containing 75 mg of collagenase type II (Worthington, Lakewood, NJ) at 37°C with rapid shaking. After digestion, the cells were washed once with 10 ml of DMEM supplemented with 10% bovine calf serum. The cells were plated into 75-cm² flasks and allowed to adhere to the flasks overnight. The next day the cells were treated with 50 ng/ml of DT for 24 hours, followed by aspiration of media and replacement of media with DMEM supplemented with 10% bovine calf serum. In order to demonstrate that DT treatment causes elimination of melanoma cells, cell cultures were analyzed before and after DT treatment by Western blot, using an antibody to microphthalmia, a transcription factor expressed specifically in melanocytes and retained in melanoma cells.^{19,20} For the Western blot, cells were washed with PBS and treated with sodium dodecyl sulfate (SDS) extraction buffer (250 mmol/L Tris, 4% SDS, 20% glycerol) and immediately boiled. Protein extracts were resolved using an 8.5% SDS PAGE gel and transferred to nitrocellulose. Prestained standards were used (Gibco/BRL, Grand Island, NY). The Western blot was analyzed simultaneously with anti-Microphthalmia (obtained from David Fisher, Dana Farber Cancer Institute, Boston, MA)²⁰ and mouse anti-alpha tubulin antibodies (Sigma) and subsequently with horseradish peroxidase conjugated goat anti-mouse antibody as the secondary antibody. Enhanced chemiluminescence signal was visualized on Amersham Hyperfilm ECL film. B16 melanoma cells were used as a standard for the microphthalmia protein, which routinely runs as a doublet between 52 and 56 kd.

DT Toxicity Assay

DT toxicity assays on MMAN cells were performed according to the method of Raab et al.¹⁸ Briefly, 1×10^5 cells/well were plated in 96-well plates. Twenty-four hours after plating, cells were exposed to increasing concentrations of DT for 90 minutes. The medium was removed and the cells were incubated with [³H] leucine in RPMI 1640 leucine-free media (Gibco, 0.005 mCi/ml) for 60 minutes. The cells were harvested with trypsin, transferred to filters, and the extent of [³H]-leucine incorporated into protein was measured with a liquid scintillation counter (MicroBeta Plus, Wallac, Hamden, CT). Each point was performed in triplicate and results normalized to 100%, which represents the counts per minute of incorporated [³H]-leucine obtained in the absence of DT.

Immunohistochemistry

After DT treatment of melanoma tumors, cells were fixed with 4% paraformaldehyde and immunostained for the presence of the smooth muscle-specific marker calponin. Anti-calponin (mouse monoclonal Ab provided by Marina

Figure 1. Parental mouse 32D myeloid cells grown in suspension (open circles), and 32D cells transfected with human HB-EGF cDNA (closed circles) were treated with increasing concentrations of DT and incorporation of [3 H]-leucine into protein was measured. Each point was performed in triplicate and the results were normalized to the cpm of incorporated [3 H]-leucine obtained in the absence of DT.

Glukhova, Curie Institute, Paris) was used at 1:50. All antibody-antigen complexes were visualized using the Vectastain Elite ABC Kit (Vector, Burlingame, CA) and the biotinylated anti-mouse secondary antibody provided by the manufacturer. Staining of endothelial cells was performed by addition of dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) to culture media to a final concentration of 10 μ g/ml and observation with a rhodamine filter according to the method of Voyta et al.²¹

Quantitation of stromal cell content *in vivo* was performed by smooth muscle actin staining of paraffin sections of MMAN melanoma tumors in nude mice. Five-micron sections of formalin-fixed, paraffin-embedded tissue were immunostained for expression of smooth muscle actin (prediluted, Ventana, Tucson, AZ). An avidin-biotin complex enzyme kit (Signet Laboratories, Dedham, MA) with steam heat-induced epitope antigen retrieval was used in combination with the Dako Autostainer (Dako, Carpinteria, CA). Hematoxylin was used as a counterstain.

The percentage of stroma compared to the tumor component was quantitated in 20 high-power fields (40 $\times$ magnification lens, 10 $\times$ magnification eye piece) using a 100-point graduated counting grid. The number of cross points which covered an immunostained stromal area was counted. This stromal area accounted for a mean of 1.5% of the total area of tumor.

RNase Protection Assay

RNA was extracted with RNAzol B (Tel-Test, Friendswood, TX) from mouse liver, cultured MMAN cells, MMAN tumor xenografts, or cultured tumor stromal cells resulting from the treatment of dispersed MMAN tumors with DT. A plasmid containing a 512-bp segment of mouse VEGF₁₈₈ (Ng, Rohan, and D'Amore, unpublished data) was used to generate a 32 P-labeled antisense riboprobe per manufacturer's protocols (Ambion, Austin, TX). This riboprobe can detect all known VEGF mRNA isoforms.²² Hybridization of the riboprobe with murine VEGF₁₈₈

Figure 2. DT killing of human melanoma cells in culture. MMAN melanoma cells were incubated with increasing amounts of DT. Protein synthesis was determined as in Figure 1.

mRNA results in a 512-nucleotide protected fragment. A secondary protected fragment of 400 nucleotides, due to internal cleavage at a poly-A-rich region in VEGF₁₈₈, was also observed in mouse liver. Hybridization of the riboprobe with murine VEGF₁₆₄ and VEGF₁₂₀ mRNAs results in protected fragments of 333 and 201 nucleotides, respectively. Hybridization of the murine probe with human VEGF mRNA does not result in a protected fragment due to numerous internal mismatches. The RNase protection assays were performed according to the method of Hod.²³ Protected fragments were separated on gels of 5% acrylamide, 8 mol/L urea, 1 $\times$ Tris-borate buffer, and quantified with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Simultaneous hybridization with a 32 P

Figure 3. DT treatment of a human melanoma ablates microphthalmia protein levels. Melanoma tumors were cultured in the absence (lane 1) or presence (lane 2) of DT. A mouse B16 melanoma cell lysate was used as a positive control (lane 3). Western blot analysis of the lysates was carried out with anti-microphthalmia and anti-tubulin antibodies. The upper doublet represents microphthalmia protein (bracket), whereas the lowest band represents tubulin, which was used as a control for loading.

Figure 4. Morphology and immunohistochemistry of tumor stromal cells. **A:** An MMAN xenograft was treated with collagenase and cells were plated in tissue culture flasks. **B:** The same culture in **A** observed 2 days after treatment with 100 $\mu\text{g/ml}$ DT. **C:** Immunohistochemical staining of calponin, a smooth muscle marker. **D:** Immunofluorescent staining with diI-Ac-LDL, a marker of endothelial cells. The stromal cells were confluent at the time of photography, and the LDL-positive cells comprise 1 to 4% of the total cells.

labeled riboprobe for mammalian 18S ribosomal RNA (Ambion) was done in order to normalize for variations in loading and recovery of RNA. The amount of radioactivity in each protected fragment was further normalized to the length of the protected fragment.

Results

DT Is Toxic for Mouse Cells If They Express Human HB-EGF/DTR

Parental mouse 32D myeloid cells and 32D cells expressing the human HB-EGF were incubated with increasing concentrations of DT (Figure 1). The mouse 32D cells were unaffected by DT treatment even at 100 ng/ml. On the other hand, the human HB-EGF mouse cell transfectants were killed by DT at a half-maximal dosage of 5 ng/ml. Thus, expression of human HB-EGF confers DT sensitivity on murine cells.

DT Is Toxic to MMAN Human Melanoma Cells in Vitro and in Vivo

MMAN human melanoma cells express HB-EGF/DTR (not shown). These cells were cultured with increasing con-

centrations of DT (Figure 2). DT killed MMAN cells in a dose-dependent manner, as measured by inhibition of protein synthesis with an ED_{50} of 40 ng/ml.

Because murine cells are resistant to DT and human cells expressing HB-EGF/DTR are sensitive to DT, it should be possible to kill human tumors grown in mice without adversely affecting the animals. MMAN cells form invasive tumors in nude mice. Accordingly, these cells were injected subcutaneously into mice. When MMAN cells were injected into groups of 4 mice per experimental condition nude mice, large melanoma tumors over 1 cm^3 in size were detected within 4 weeks. Treatment of these animals containing large established tumors with a single intraperitoneal injection of 1 μg DT per animal led to rapid and total regression of tumors within 7 days (data not shown). Thus, the sensitivity of MMAN melanoma cells to DT *in vivo* is consistent with the effects of DT on these cells *in vitro*. After tumor regression, the mice survived for at least 3 months with no weight loss or other detectable toxic effects.

DT Treatment Eliminates Tumor Cells from Mixed Tumor/Stromal Cultures

In order to show that DT was highly effective in killing human tumor cells, Western blot analysis was carried out

with an antibody directed against microphthalmia, a transcription factor found primarily in melanocytes and melanoma cells and therefore a useful melanoma marker (Figure 3).^{19,20} MMAN tumors were excised and treated with DT. After DT treatment, the levels of microphthalmia protein were undetectable, suggesting that the human tumor cells had been eliminated.

Murine stromal cells surviving DT treatment were analyzed for expression of endothelial cells and smooth muscle cell markers (Figure 4). Figure 4A shows a culture of cells derived from an MMAN tumor and Figure 4B shows the murine cells that survived DT treatment. Surviving murine stromal cells contained smooth muscle cells as determined by positive immunostaining for calponin, a smooth muscle marker (Figure 4C), and a mixture of endothelial cells and macrophages as determined by immunofluorescence with dil-Ac-LDL,²¹ a marker for both of these cell types (Figure 4D).

The distribution of stromal cell types within the MMAN tumor was estimated in several ways. By immunostaining, approximately 90% of the cells that remained in culture after DT treatment were found to be calponin-positive, indicating that the vast majority of tumor stromal cells are of smooth muscle origin. The remaining 10% of the stromal cells were LDL-positive, and likely constitute a mixed population of endothelial cells and macrophages. The content of stromal cells was also analyzed by flow cytometry of trypsinized stromal cells. Approximately 4% of the stromal cells were LDL-positive and stained with von Willebrand factor, a specific marker of endothelium (data not shown). The lower estimate of non-smooth muscle stromal cell content in this analysis was most likely due to a lack of macrophages in the cell sort because these cells are resistant to trypsinization. In estimating the stromal cell content of the MMAN tumor, smooth muscle actin staining of tumor sections indicated that smooth muscle cells, which constitute the vast majority of stromal cells, made up approximately 1.5% of the cells in the tumor.

Tumor Stromal Cells Express VEGF mRNA in Vitro

VEGF is a potent angiogenesis factor that has been shown to induce tumor neovascularization.^{24–29} MMAN tumors consist mostly of tumor cells as compared to the infiltrating murine stromal cells. Because human MMAN cells express VEGF (not shown), a species-specific RNase protection assay was developed (Figure 5). This RNase protection assay detects the three murine VEGF mRNA isoforms (VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀) but not human VEGF mRNA. The relative distributions of VEGF isoforms were quantitated after normalization to the length of the protected fragment. VEGF₁₈₈ and VEGF₁₆₄ are the predominant mRNA isoforms detected in the adult mouse liver (Figure 5, lane 3) comprising 41% and 43%, respectively, of the total VEGF mRNA. In the stromal cell cultures, VEGF₁₆₄ and VEGF₁₂₀ mRNAs comprise 50% and 43%, respectively, of the total VEGF mRNA (Figure 5, lane 6). As expected, murine VEGF mRNA was not detected in the cultured MMAN tumor cells (Figure 5, lane

Figure 5. RNase protection assay using a murine-specific probe for the three VEGF mRNA isoforms. RNA from the indicated sources was hybridized to ³²P-labeled riboprobes for murine VEGF and mammalian 18S. Samples were either untreated (–) (lane 1) or treated with RNases (+) (lanes 2–6) and electrophoresed in a denaturing acrylamide gel. The full-length ³²P VEGF and 18S probes are indicated by arrows at left. The protected fragments (VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀) resulting from hybridization to each of the VEGF mRNA isoforms are indicated by arrows at right. Lanes 1–2: incubation of ³²P-labeled VEGF with control tRNA in the absence or presence of RNase. Lanes 3–6: The protected fragments from mouse liver, an MMAN tumor, cultured MMAN cells, and stromal cells derived after DT treatment of an MMAN tumor, respectively. The protected fragment in mouse liver (lane 3) between VEGF₁₈₈ and VEGF₁₆₄ is an artifact that results from internal cleavage of the VEGF₁₈₈ hybrid at a Poly-A-rich region.

5). Murine VEGF also was not detected in the MMAN tumor xenografts which have a mixed cell population of human tumor cells and murine stromal/endothelial cells (Figure 5, lane 4). The inability to detect murine VEGF mRNA in the whole MMAN tumor is attributable to the low percentage of stromal cells in the tumor. Only when the stromal cells are concentrated was murine VEGF mRNA detected.

Discussion

The ability to study tumor cell-stromal cell interactions has been greatly hindered by the inability to separate host stromal cells from tumors. There is a report that endothelial cells have been isolated from the Rif V fibro-

sarcoma, by flow cytometry for acetylated LDL.³⁰ However, this technique does not result in isolation of non-endothelial stromal cells, which may play a major role in facilitating tumor growth. To circumvent this problem, we have used differential DT toxicity to facilitate the isolation of tumor stromal cells in a single, rapid step. DT has the unusual ability to kill cells of nearly all species other than mice or rats. This selectivity has been shown to be due to lack of binding of DT to the mouse homologue of the DT receptor, HB-EGF.^{14,15} This method of stromal cell isolation should be applicable to all human tumors expressing HB-EGF/DTR or to other types of xenografts, such as human skin involved in pathogenic processes.^{31,32}

We have been able to isolate stromal cells from a human melanoma (MMAN) tumor by selectively killing the human tumor cells with DT, leaving viable murine stromal cells that can be cultured. In the case of a melanoma tumor, about 90% of the stromal cells are smooth muscle cells, while the rest are mostly endothelial cells and macrophages. The stromal smooth muscle cells constitute between 1.5 and 2% of the total tumor cell population, as determined by smooth muscle actin staining.

To confirm the murine origin of the viable stromal cells remaining after DT treatment, we analyzed VEGF mRNA expression, because there have been previous reports (for example using green fluorescent protein methods) that tumor stromal cells express elevated levels of VEGF.³³ An RNase protection assay with a murine-specific VEGF probe detected expression of both the murine VEGF₁₂₀ and VEGF₁₆₄ isoforms by stromal cells.

The ability to readily isolate and culture primary stromal cells will facilitate analysis of factors involved in recruitment of stromal cells, for example PDGF BB,³⁴ angiopoietin-1³⁵ and VEGF. Presently, these growth factors are typically analyzed for their effects on cell lines. In addition, the analysis of stromal cell-derived growth factors and the regulation of their expression will be facilitated. Furthermore, if stromal cells and their growth factors are important contributors to tumor growth, then inhibition of stromal cell recruitment may be an effective target for drug therapy of solid tumors.

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