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Production of Transforming Growth Factor Alpha by Hamster Eosinophils

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Previously it was demonstrated that malignant transformation of the Syrian hamster cheek pouch mucosa is associated with the expression of TGF-α. Therefore* in situ *hybridization and immunohistochemistry was used to investigate the cellular sources of TGF-α production in this model system. Surprisingly one cell type in the inflammatory infiltrate present in the connective tissue adjacent to the transformed epithelium represented a major source of TGF-α mRNA. Detailed analysis of these cells revealed that they were eosinophils. In addition to TGF-α mRNA, about 40% of the eosinophils associated with the oral tumors exhibited TGF-α product reactive with a monoclonal antibody against the C terminus of the mature TGF-α peptide. Normal hamster bone marrow eosinophils also exhibited TGF-α mRNA and product by* in situ *hybridization and immunohistochemistry. These results suggest that the eosinophil represents a biologically significant source of TGF-α. (Am J Pathol 1990, 137:1425–1434)

Transforming growth factor alpha (TGF-α) is a 50-amino acid peptide that was initially defined as an activity, present together with TGF-β in retrovirus-transformed cells, that can compete with epidermal growth factor (EGF) for the binding to the EGF receptor.[1] Subsequent work showed that most malignant epithelial tumors express TGF-α.[2] Through its action on the EGF-receptor, TGF-α is believed to induce epithelial cells to express transformed phenotypes through autocrine mechanisms.[3] While it is believed that the transformed epithelium is the major source of the detected TGF-α in malignant epithelial tumors, TGF-α recently also was demonstrated in certain normal tissues and cell types, including normal human keratinocytes,[4,5] bovine anterior pituitary cells,[6] rat maternal decidua,[7] mouse blastocysts,[8] and macrophages.[9,10] These findings suggest that TGF-α may have a physiologic role in normal tissues, possibly through autocrine and/or paracrine mechanisms.[11] No cell type of hematopoietic origin, with the exception of the macrophage,[9,10] has been found to produce this cytokine.

We investigated the expression of TGF-α during the development of experimental cancers in the cheek pouch of the Syrian hamster. The hamster cheek pouch represents an excellent setting for the study of oral carcinogenesis because specific carcinogenic chemicals, such as 7,12-dimethylbenz[*a*]anthracene (DMBA), consistently induce transformation-associated lesions (hyperplasia, dysplasia, squamous cell carcinoma) in a time-dependent manner.[12] Recently we demonstrated that the chemically induced malignant transformation of oral mucosal tissue in the Syrian hamster cheek pouch is associated with the aberrant expression of the TGF-α gene.[13] In this study, we investigated the cellular sources of TGF-α in this system using *in situ* hybridization and immunohistochemistry.

This paper presents the unexpected finding that a major source of TGF-α mRNA and product in DMBA-transformed hamster oral epithelium is the inflammatory infiltrate adjacent to the developing carcinoma. Cytochemical and physical characterization reveals that the only connective tissue cells detectably positive for TGF-α mRNA are eosinophilic granulocytes. These cells also contain TGF-α product, as detected by immunohistochemistry, with a

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monoclonal antibody directed against the C terminus of the mature TGF-α peptide. Furthermore the eosinophils present in normal hamster bone marrow also contained TGF-α mRNA and protein.

Materials and Methods

DMBA-induced Hamster Cheek Pouch Tumors

Epidermoid carcinomas were induced in the cheek pouches of 10 male Syrian hamsters according to the protocol of Shklar.[12] Briefly 0.5% DMBA in mineral oil was applied three times weekly to the left cheek pouch of each experimental animal for 14 weeks. All animals developed grossly exophytic tumors. Exophytic tumors larger than 10 mm in diameter were excised and a sample of each was saved for histopathologic examination. The remaining tissue was immediately processed for *in situ* hybridization, as described below.

Normal Hamster Bone Marrow Preparation

Four normal male Syrian hamsters (about 90 days old) were killed by cervical dislocation. Four long bones from each animal (two femura and two humeri) were harvested and the marrow contents of each was flushed with 5 ml of ice-cold 1× Hanks buffer. The pooled bone marrow from each animal was centrifuged at 2000*g* for 20 minutes at 4°C. Each of the resultant pellets was resuspended into 2 ml of ice-cold 1× Hanks and then subjected to a double-gradient procedure to isolate the granulocyte population. Briefly the double gradient was formed by layering an equal volume (2 ml) of Histopaque-1077 over 2 ml of Histopaque-1119 (Sigma Chemical Co., St. Louis, MO). The bone marrow mixture (2 ml in 1× Hanks) was layered carefully onto the upper histopaque-1077 medium. The tubes were centrifuged at 700*g* for 30 minutes at room temperature. Cells of the granulocytic series accumulate at the 1077/1119 interphase; ~5% of these cells were eosinophils. The cells in this layer were washed twice in ice-cold 1× PBS and then resuspended into 1 ml of 1× PBS. This was then embedded into 1 ml of 1% agar in 1× PBS. The agar pellet was immediately fixed in freshly prepared 4% paraformaldehyde and then processed for *in situ* hybridization, as described below.

In Situ *Hybridization*

DMBA and mineral oil-treated hamster cheek pouch tissues were processed for *in situ* hybridization according the method of Zeller et al,[14] with minor modifications.[15] Excised hamster cheek pouches were fixed immediately in freshly prepared 4% paraformaldehyde in 1× PBS (4 g/100 ml) for 2 hours at 4°C. Then they were dehydrated through increasing percentages of ethanol, then xylene, and finally embedded into paraplast wax. Eight-micron sections were cut and mounted onto gelatin-coated glass slides. Before hybridization, each tissue section was pretreated and prehybridized. Pretreatment included tissue rehydration, mild acid hydrolysis, postfixation of tissues with 4% paraformaldehyde, and finally treatment in 0.25% (v/v) concentration of acetic anhydride in 0.1 mol/l (molar) triethanolamine (pH 8). Prehybridization of the pretreated tissue section was a 2-hour incubation at 42°C of the tissue sections with sufficient quantities (~25 μl/section) of the hybridization solution (containing S-UTP–labeled pGEM3 single-stranded RNA; 50% deionized formamide; 0.3 mol/l NaCl; 10 mmol/l (millimolar) TRIS HCl pH 8.0; 1 mmol/l EDTA pH 8.0; 1× Denhardt's solution; 500 μg/ml tRNA; 500 μg/ml poly[A]; 50 mmol/l dithiothreitol; and 10% polyethylene glycol) without the probe. Hybridization of the tissue sections was performed immediately after pretreatment and prehybridization. ^{35}S-labeled antisense (−) and sense (+) single-stranded (ss) RNA probes to the human TGF-α cDNA were used. The ss-RNA probes were added directly to the prehybridization solution on the glass slide (2×10^6 cpm per section), mixed well, and then incubated overnight at 50°C under a glass cover slip, and sealed with rubber cement. Posthybridization washings followed the overnight hybridization and included a RNase digestion step: 15 minutes twice in 50% formamide, 2× SSC, 20 mmol/l 2-mercaptoethanol, at 50°C; 15 minutes twice in 50% formamide, 2× SSC, 20 mmol/l 2-mercaptoethanol, 0.5% Triton X-100, at 50°C; 5 minutes twice in 2× SSC, 20 mmol/l 2-mercaptoethanol, at room temperature; RNase digestion for 30 minutes at 37°C: 40 μg/ml of RNase A, 2 μg/ml RNase T1, 10 mmol/l TRIS HCl pH 7.5, 5 mmol/l EDTA, 0.3 mol/l NaCl; 15 minutes in 2× SSC, 20 mmol/l 2-mercaptoethanol, at room temperature; four times each 30 minutes in 2× SSC, 20 mmol/l 2-mercaptoethanol, at 50°C with gentle shaking; and once for 10 minutes in 2× SSC at room temperature. The slides then were dehydrated, dipped in Kodak NTB2 emulsion, and processed for autoradiography (Kodak, Rochester, NY). All slides were counter stained with Giemsa (Fisher #SG-28, Springfield, NJ).

Two reports in the literature have indicated that eosinophils can bind DNA[16] or RNA[17] probes nonspecifically during *in situ* hybridization. In preliminary work we defined carefully experimental conditions (described above) that permit specific labeling of eosinophil mRNA by *in situ* hybridization. Hybridization at temperatures less than 50°C and/or durations less than 12 hours resulted in nonspecific eosinophil labeling. The use of paraffin-embedding of paraformaldehyde fixed tissues, incorporation of a pre-

hybridization step with a solution containing S-UTP–labeled cold probe for at least 2 hours at 42°C, and the use of RNase A and T1 in posthybridization washing are important for eliminating such nonspecific binding. Finally the appropriate use of sense-riboprobe controls, heterologous riboprobe controls, and positive and negative control tissues also help to establish confidence in the interpretation of results.

Molecular Probes

The human TGF-α (hTGF-α) cDNA was provided by G. I. Bell of Chiron Corporation (Emeryville, CA). This cDNA was recloned into plasmid vectors pGEM3 and pGEM4 (Promega, Madison, WI). The hTGF-α cDNA of the desired orientation (sense or antisense) was cloned downstream to the T7 promoter. The rat histone H3 cDNA probe was provided by Dr. William F. Marzluff.[18] This cDNA also was recloned into the plasmid vector pGEM3 (Promega) such that antisense transcripts will be generated by the T7 promoter. Production of labeled riboprobes was done using a RNA transcription kit (Promega, Madison, WI), ^{35}S-UTP (Amersham, SJ.40383, SP6/T7 Grade, 850 Ci/mole, Arlington Heights, IL), and T7 RNA polymerase (Stratagene, La Jolla, CA). Typically 80% to 90% incorporation was obtained. Each *in vitro* transcription reaction yields ~230 ng of synthesized RNA with a specific activity of ~3 × 10^8 cpm/μg RNA. These probes were used for hybridization without alkaline hydrolysis.[14] The final specific activity of the probe used for *in situ* hybridization was ~2 × 10^6 cpm per section.

Immunohistochemistry

Six-micron sections of tumor-bearing hamster cheek pouch preparations embedded in paraffin were subjected to staining for TGF-α protein using a monoclonal antibody (MAb) directed against the C terminus (residues 34-50) of the mature 50-amino acid human TGF-α peptide (TGF-α: Ab-2; GF-10; Oncogene Science, Mahasset, NY). Eighteen micrograms per milliliter of the MAb was used to stain each section. A control monoclonal antibody to the bacterial protein β-galactosidase (Ab-1; OBO2; Oncogene Science) at the same concentration was used as negative control. For signal detection, we used the Vectastain ABC-Alkaline Phosphatase Kit (Mouse IgG AK-5002, Vector Laboratories, Burlingame, CA) in conjunction with the alkaline phosphatase substrate kit I (SK-5100) in the presence of 1.25 mmol/l of levamisole (SP-5000). Sections were counterstained with 0.2% aniline blue for 10 minutes to identify eosinophils.[19]

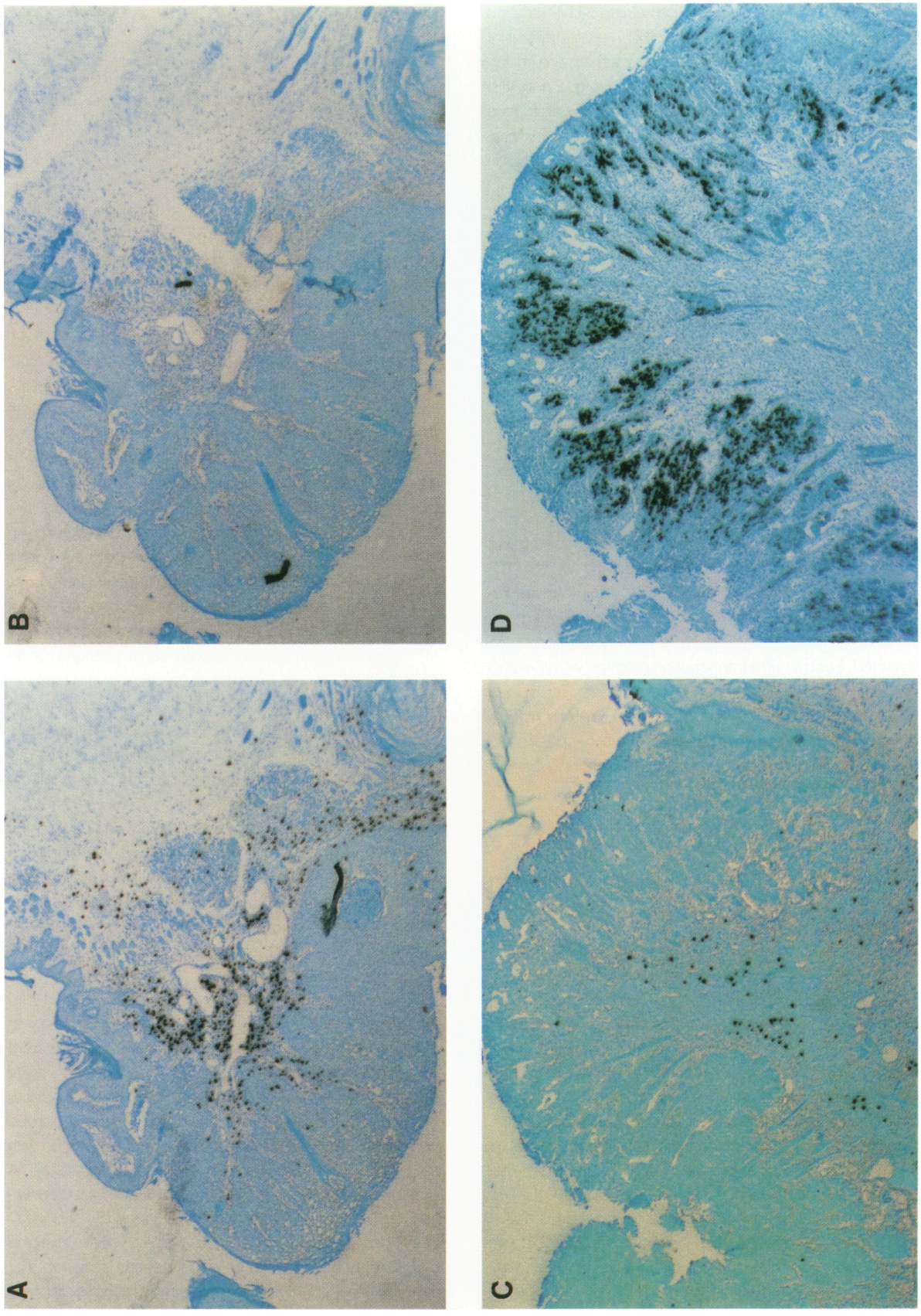
Results

Cellular Elements Within the Inflammatory Infiltrate Associated with DMBA-induced Hamster Oral Cancers Contain High Levels of TGF-α mRNA

In situ hybridization was used to localize the cellular sources of TGF-α mRNA in normal and DMBA-transformed hamster oral epithelium. As described before,[15] both normal and DMBA-treated hamster oral epithelium contained low but detectable levels of TGF-α mRNA with the normal oral epithelium exhibiting approximately one half as much TGF-α mRNA as hyperplastic, dysplastic, or carcinomatous epithelium (data not shown). Surprisingly certain cellular elements associated with the inflammatory infiltrate that typically develops near DMBA-induced tumors expressed high levels of TGF-α mRNA as detected by *in situ* hybridization (Figure 1A). Each of the 10 tumor-bearing pouches examined contained such TGF-α mRNA-positive lamina propria cells, which typically were associated with either moderate to poorly differentiated portions of the tumor and/or with the tumor's blood supply. Sometimes TGF-α mRNA-positive cells similar to those in the lamina propria were observed within the transformed epithelium.

Figure 1A and B demonstrate serial sections of an exophytic carcinoma from the cheek pouch of a hamster that was painted with DMBA for 14 weeks. The section in Figure 1A, hybridized with a ^{35}S-labeled antisense TGF-α riboprobe, demonstrates many cells labeling prominently with the riboprobe in the lamina propria portion of the developing carcinoma. Most of these TGF-α mRNA-positive lamina propria cells are immediately adjacent to the invading front of the malignant epithelium, in close proximity to the prominent blood vessels associated with the tumor. Hybridization of a contiguous section with a ^{35}S-labeled sense TGF-α riboprobe as a control (Figure 1B) did not result in the labeling of any cell, either in the epithelium or lamina propria.

To further demonstrate the specificity of our *in situ* hybridization procedure, we labeled a section containing TGF-α mRNA-positive lamina propria cells with a different riboprobe, the rat histone H3. Figure 1C shows an invasive portion of a 14-week DMBA-treated hamster oral cancer probed with the ^{35}S-labeled antisense TGF-α riboprobe demonstrating TGF-α mRNA-positive cells associated with the cellular infiltrate beneath the malignant epithelium. A contiguous section was probed with the ^{35}S-labeled antisense rat histone (H3) riboprobe (Figure 1D) shows labeling only of the highly proliferative portion of the invading malignant epithelium. No cells in locations similar to that of the TGF-α mRNA-positive lamina propria cells were labeled by the H3 riboprobe.


B
D
A
C

Identification of the TGF-α mRNA-positive Lamina Propria Cells as Eosinophils

Exposures of the *in situ* hybridization autoradiograms shorter than those shown in Figure 1 revealed that the TGF-α mRNA-positive lamina propria cells had a bilobed or polylobed nuclei and prominent cytoplasmic granules. This prompted us to investigate if the TGF-α mRNA-positive lamina propria cells originate from leukocytes. Serial sections of DMBA-induced hamster oral cancers containing TGF-α mRNA-positive lamina propria cells were subjected to *in situ* hybridization with the ^{35}S-labeled antisense TGF-α riboprobe and counterstained with a Giemsa stain preparation that differentiates different granulocytes (Rowley Biochemical Institute, Rowley, MA). Figure 2A is a high-power view of one such DMBA-induced hamster oral cancer containing many TGF-α mRNA-positive lamina propria cells. The light pink-purple color of the cytoplasmic granules of these cells indicated that they might be eosinophils. Figure 2B is a an adjacent section stained only with the Giemsa preparation, exhibiting many cells with bilobed or polylobed nuclei and magenta-stained cytoplasmic granules. Examination of these cells under higher power (oil, 1250×) revealed the oblong cytoplasmic granules typical of eosinophils.

To confirm further the histochemical identification of the TGF-α mRNA-positive lamina propria cells as eosinophils, we took advantage of the fact that eosinophils exhibit a much more intense natural autofluorescence than any other nucleated cell.[20] We found that staining of eosinophils with a Fisher Giemsa stain (SG-28) followed by viewing with rhodamine filters at 552 nm gave rise to a brilliant red-orange fluorescence in the cytoplasm of these cells. This is demonstrated in Figure 2C to F. Figure 2C is a bright-field view of the lamina propria portion of a 14-week DMBA-treated hamster cheek pouch tumor hybridized with a ^{35}S-labeled antisense TGF-α riboprobe, demonstrating two nucleated cells labeling prominently with this riboprobe. Note that the mast cells in this section, which exhibit basophilic cytoplasm, are not labeled with the ^{35}S-labeled antisense TGF-α riboprobe. Figure 2D is the dark-field visualization, using a green filter of the same section shown in Figure 2C. The autoradiographic grains appear green. Figure 2E is a fluorescence visualization of the same section using rhodamine filters at 552 nm, demonstrating prominent fluorescence of two of the cells in the field. Figure 2F is a composite exposure of the same section demonstrating that the cells labeled with the ^{35}S-labeled antisense TGF-α riboprobe and the fluorescence cells are the same.

Eosinophils Associated with Hamster Oral Cancers Contain TGF-α Product

To determine if the TGF-α mRNA detected in tumor-associated eosinophils is translated into TGF-α protein, we performed immunohistochemistry using a monoclonal antibody (MAb) directed against the C terminus (amino acids 34 to 50) of the human TGF-α mature peptide. We recently obtained the nucleotide sequence of the hamster TGF-α cDNA containing the mature peptide coding region (Chiang et al, unpublished data). The amino acid sequence between positions 34 to 50 in the hamster is identical to that in the human. Thus the hTGF-α MAb should recognize hamster TGF-α protein present in hamster eosinophils. Immunohistochemistry was performed on the 10 tumor-bearing hamster cheek pouches containing TGF-α mRNA-positive eosinophils. In accord with previous work with human epithelium,[4,5] we found that normal hamster oral epithelium exhibited a uniformly faint but definitive positivity for TGF-α throughout the epithelium and that transformed epithelium (hyperplastic, dysplastic, or carcinomatous) exhibited an intense positivity in the spinous layer but faint reactivity in the proliferating basal layers and keratin layers (data not shown). We also observed that lamina propria portions of DMBA-transformed hamster oral cancers contained TGF-α immunoreactive cells in a distribution very similar to that of the TGF-α mRNA-positive cells.

To determine if these TGF-α immunoreactive cells were eosinophils, the sections were counterstained with a 0.2% aniline blue solution. Eosinophil cytoplasmic granules stained with this dye emit a blue fluorescence when viewed with a DAPI filter at 365 nm.[19] Furthermore we found that the aniline blue fluorescence of eosinophil cytoplasmic granules is not abolished by the red substrate staining of our immunohistochemical procedure. As a result, both procedures can be used on the same preparation to determine specifically whether eosinophils contain detectable TGF-α protein.

Approximately 40% of all aniline blue fluorescent eosinophils were stained by the hTGF-α MAb. No nonfluorescent cells (ie, neutrophils and mononuclear cells) were detectably stained by the hTGF-α MAb. Staining of similar preparations with the control MAb (bacterial β-galactosidase) resulted in no detectable staining of eosinophils or

Figure 1. *Detection of TGF-α mRNA in DMBA-induced hamster oral cancers by* in-situ *hybridization.* **A:** *A hamster cheek pouch oral carcinoma (14 weeks DMBA treatment) hybridized with a ^{35}S-labeled antisense TGF-α riboprobe.* **B:** *A section contiguous to that in* **A** *hybridized with a control ^{35}S-labeled sense TGF-α riboprobe.* **C:** *a different hamster cheek pouch oral carcinoma (14 weeks DMBA treatment) hybridized with a ^{35}S-labeled antisense TGF-α riboprobe.* **D:** *A section contiguous to that in* **C** *hybridized with a ^{35}S-labeled antisense rat histone H3 riboprobe. All sections were counter-stained with Giemsa preparation (see Materials and Methods), exposure time for autoradiography was 3 days at 4°C, original magnification 31×.*

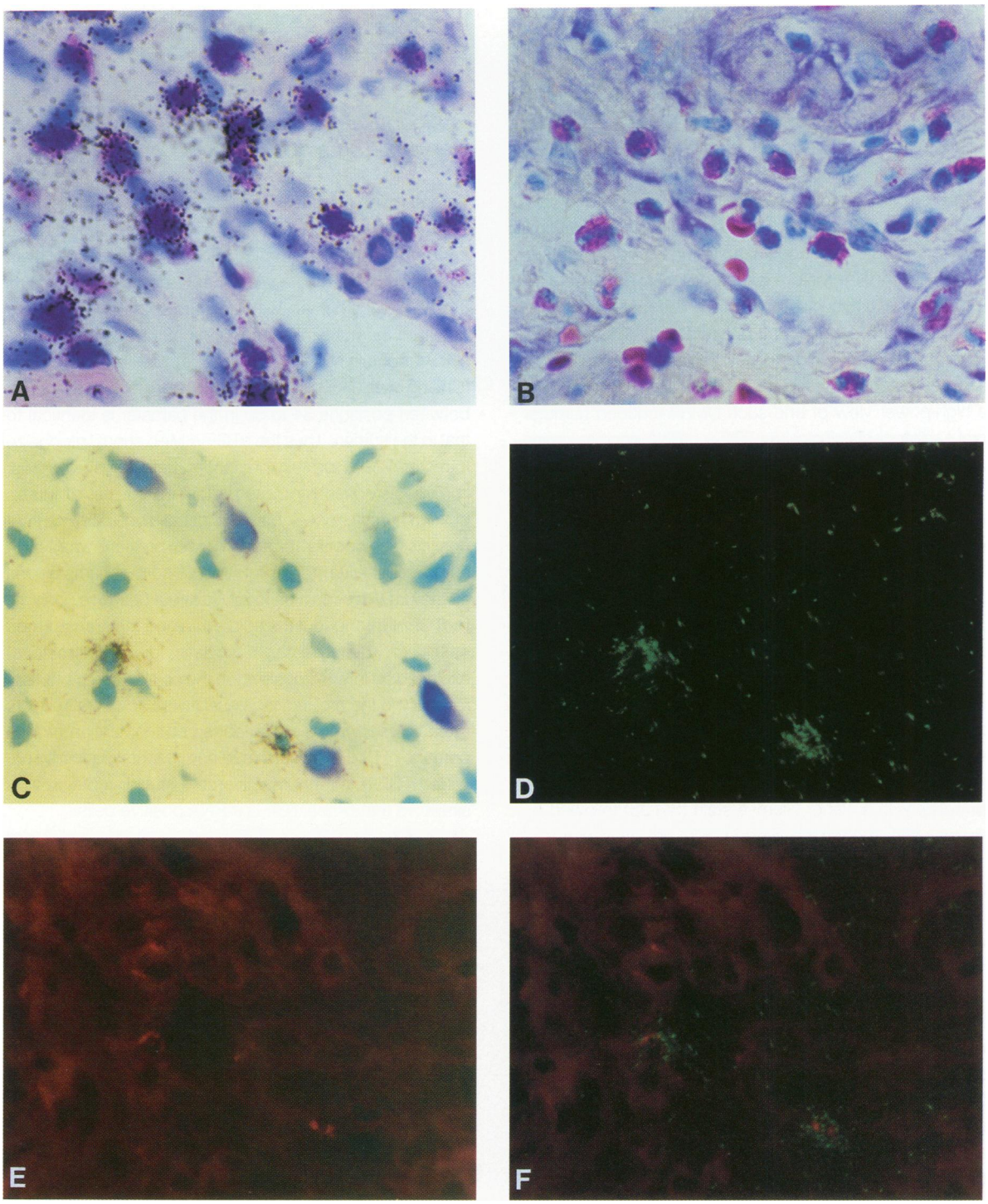


Figure 2. *The TGF-α mRNA-positive lamina propria cells in DMBA-induced hamster oral carcinomas are eosinophils.* **A:** *High-power examination at the lamina propria portion of a 14 week DMBA treated hamster cheek pouch carcinoma demonstrating many TGF-α mRNA-positive lamina propria cells as detected by hybridization with the ^{35}S-labeled antisense TGF-α riboprobe.* **B:** *A Giemsa-stained section contiguous to that shown in* **A**, *but without hybridization demonstrating many eosinophils. The sections in* **A** *and* **B:** *original magnification 1250×, were counter-stained with a Giemsa preparation from Rowley Biochemical Institute (Rowley, MA).* **C:** *bright-field view of the lamina propria portion of a 14 week DMBA-treated hamster cheek pouch tumor hybridized with a ^{35}S-labeled antisense TGF-α riboprobe. The cells with basophilic cytoplasm are mast cells.* **D:** *Dark-field visualization of the same section shown in* **C** *using a green filter to highlight the autoradiographic grains.* **E:** *Fluorescence visualization of the same section shown in* **C** *using rhodamine filters at 552 nm to demonstrate two fluorescent cells.* **F:** *A composite exposure of the section shown in* **C**, *demonstrating that the cells labeled with the ^{35}S-labeled antisense TGF-α riboprobe and the fluorescent cells are identical.*

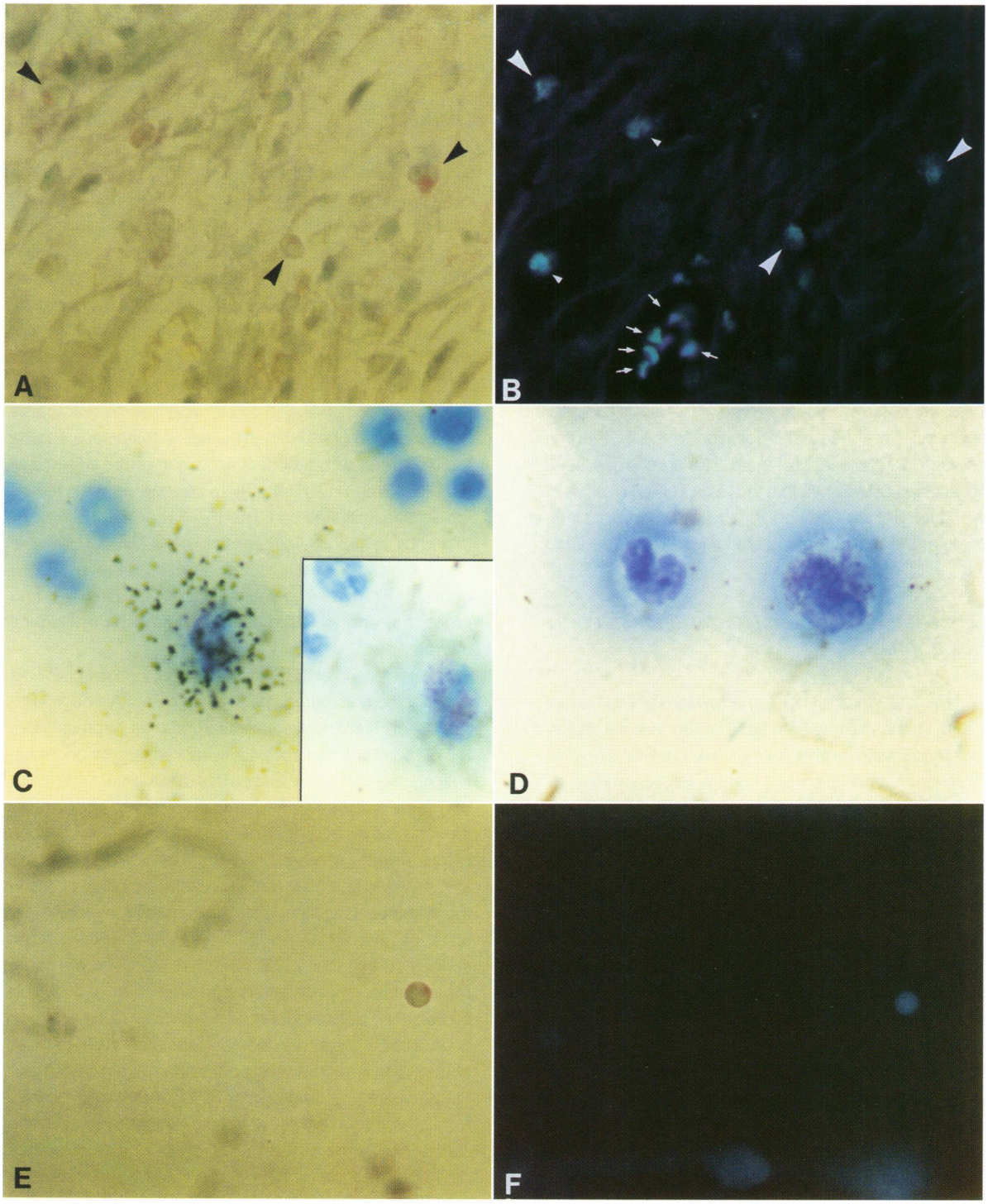


Figure 3. *Detection of TGF-α in hamster eosinophils.* **A** *and* **B**: *Immunohistochemical detection of TGF-α product in eosinophils associated with DMBA-induced hamster oral carcinomas. The counter stain is 0.2% aniline blue. Original magnification, 400×.* **A**: *Bright-field photomicrograph at the lamina propria portion of a hamster cheek pouch tumor treated with DMBA for 14 weeks and stained with the TGF-α MAb (18 μg/ml) followed by detection with an alkaline phosphatase method. The color of the substrate reaction is red. Three cells in the field demonstrate prominent red substrate positivity in the cytoplasm (large arrow heads).* **B**: *DAPI fluorescent visualization of the same field as in* **A**, *demonstrating aniline blue fluorescence of eosinophils (large and small arrow heads) and erythrocytes (arrows).* **C** *and* **D**: *Detection of TGF-α mRNA in normal hamster bone marrow eosinophils. Counter stain is Giemsa (Fisher). Original magnification: 1000×.* **C**: *Labeling of a normal hamster bone marrow preparation with the ^{35}S-labeled antisense TGF-α riboprobe. The photomicrograph was shot by focusing on the autoradiographic grains at the cell in the middle of the field labeled. The inset is a photomicrograph of the same area focused to demonstrate the morphologic details of the labeled cell.* **F**: *A contiguous normal hamster bone marrow section hybridized with the sense TGF-α riboprobe. The cell on the right is morphologically an eosinophil. Neither one of these two cells labeled with the riboprobe.* **E** *and* **F**: *Immunohistochemical detection of TGF-α protein in normal hamster bone marrow eosinophils. The counter stain of 0.2% aniline blue. Original magnification: 400×.* **E**: *Bright-field photomicrograph of a bone marrow preparation stained with the TGF-α MAb (18 μg/ml) followed by detection with an alkaline phosphatase method. One of the two cells in the field demonstrates red substrate positivity.* **F**: *DAPI fluorescent visualization of the same field as in* **E**, *demonstrating blue fluorescence of the TGF-α–positive cell.*

any other cells. Figure 3A and B show a section stained with the hTGF-α MAb detected with an alkaline phosphatase method. Figure 3A is a bright-field photomicrograph showing that some of the cells in the field are positive for the TGF-α product (large arrow heads). Figure 3B is the same field viewed with DAPI filters at 365 nm. It is clear that besides eosinophils, erythrocytes also exhibit blue fluorescence (arrows). However erythrocytes are nonnucleated and can be easily distinguished from eosinophils by aniline blue staining when viewed with bright-field optics. There are five eosinophils exhibiting blue fluorescence in Figure 3B (arrow heads). Three of these five fluorescent eosinophils are identical in location to the TGF-α immunoreactive cells seen in Figure 3A (large arrow heads). This finding establishes that the three TGF-α–positive cells in Figure 3A are eosinophils and indicates that not all eosinophils contain detectable immunoreactive TGF-α.

Detection of TGF-α in Normal Hamster Eosinophils

To evaluate whether bone marrow eosinophils express TGF-α, bone marrow derived from four normal hamsters was prepared for examination of TGF-α mRNA and protein by *in situ* hybridization and immunohistochemistry.

Approximately 70% of the eosinophils in the hamster bone marrow were labeled by the ^{35}S-labeled antisense TGF-α riboprobe, although the range of TGF-α labeling intensity in individual cells varied considerably. The control ^{35}S-labeled sense TGF-α riboprobe did not label any cell in contiguous sections. Figure 3C demonstrates the typical labeling pattern of a hamster bone marrow eosinophil with the ^{35}S-labeled antisense TGF-α riboprobe, and in the inset, the same cell photographed in a different plane of section to show morphologic detail. Figure 3D is a contiguous section labeled with the ^{35}S-labeled sense TGF-α riboprobe, demonstrating that eosinophils (cell on the right) and other bone marrow cells did not become labeled with the control riboprobe.

Approximately 20% of the bone marrow eosinophils exhibited TGF-α immunoreactivity when stained with the monoclonal against the C terminus of the mature TGF-α peptide. Figure 3E demonstrates the typical staining of a normal hamster bone marrow eosinophil with the TGF-α MAb, detected with an alkaline phosphatase method. Figure 3F demonstrates that the TGF-α immunoreactive cell is an eosinophil, as determined by its blue fluorescence after staining with the aniline blue dye. In all of the bone marrow specimens examined, only the aniline blue fluorescent eosinophils demonstrated immunoreactivity with the TGF-α MAb. Furthermore staining contiguous sections with the control monoclonal antibody against bacterial β-galactosidase did not result in positive staining of any cells.

Discussion

This study identifies the hamster eosinophil as a source of TGF-α mRNA and protein. We found that all of 10 different DMBA-induced oral squamous cell carcinomas elicited an inflammatory cell infiltrate in the underlying dermis and that this infiltrate contained many cells that were markedly positive for TGF-α mRNA by *in situ* hybridization. By morphologic and histochemical criteria, virtually all of the TGF-α mRNA-positive infiltrating cells were identified as eosinophils. The specificity of the *in situ* hybridization results was demonstrated by showing that the eosinophils in adjacent sections labeled neither with the sense probe for TGF-α nor with a control antisense probe with specificity for histone H3. Furthermore eosinophils in the tumor-associated infiltrates contained TGF-α product, as determined by immunohistochemistry with a monoclonal antibody specific for the C terminus of the mature TGF-α peptide. Finally we demonstrated TGF-α mRNA and product in eosinophils present in normal hamster bone marrow.

The detection of TGF-α expression by eosinophils was unexpected and represents a novel finding. Although LPS-stimulated human alveolar macrophages express TGF-α,[9] HL-60 human promyelocytic leukemia cells and a variety of other hematopoietic cell lines lack detectable levels of TGF-α mRNA.[3] Gottlieb et al[5] demonstrated by immunohistochemistry that normal, malignant, and hyperproliferative human keratinocytes expressed TGF-α and that this cytokine also was expressed by certain cells in the infiltrates associated with inflammatory, hyperproliferative, or neoplastic skin diseases. While Gottlieb et al[5] suggested that these TGF-α immunoreactive cells might represent macrophages, Langerhans' cells, or activated T cells, their actual identity was not determined.

In both the oral carcinoma-associated infiltrates and normal bone marrow cell populations examined in our study, virtually all (more than 95%) of the cells positive for TGF-α mRNA or product could be identified as eosinophils. And some of the TGF-α mRNA-positive cells that could not be specifically identified were represented incompletely on the sections or were obscured because of large numbers of autoradiographic grains. While rare TGF-α mRNA-positive cells appeared by morphology to be macrophages, the great majority of the macrophages in our sections were negative for TGF-α mRNA. Thus eosinophils certainly represented the major population of infiltrating cells that were detectably positive for TGF-α.

On the other hand, some tumor-associated or bone marrow eosinophils lacked TGF-α mRNA or product. Approximately 70% of bone marrow eosinophils and more than 90% of tumor-associated eosinophils were positive for TGF-α mRNA by *in situ* hybridization, while the corresponding values for TGF-α immunoreactivity were about

20% and 40%, respectively. Although it is possible that other antibodies or conditions of immunohistochemistry might detect more TGF-α–positive eosinophils, it would not be surprising if the expression of TGF-α by eosinophils was subject to microenvironmental regulation. This possibility is fully consistent with information about the regulation of expression of TGF-α or other pluripotent cytokines in other cell types. For example, TGF-α expression can be detected in human alveolar macrophages stimulated with LPS but not in the unstimulated cells.[9] Similarly the expression by mouse mast cells of mRNA or product for a wide variety of cytokines is markedly augmented when the cells are stimulated *via* the IgE receptor.[21–24]

It will be of interest to search for factors that influence TGF-α expression by eosinophils and to determine whether TGF-α expression varies in eosinophils participating in different physiologic, immunologic, or pathologic responses, or according to stage of eosinophil maturation. Such studies will be important for identifying the specific signals regulating TGF-α expression and for assessing the biologic significance of eosinophil TGF-α production. However we have demonstrated that TGF-α mRNA is present in most eosinophils infiltrating 10 different chemically induced hamster oral carcinomas and in most of eosinophils in normal hamster bone marrow. This finding indicates that the expression of TGF-α by tissue eosinophils may not be an unusual occurrence. Furthermore our data indicate that at least some (about 20%) eosinophils synthesize TGF-α before leaving the bone marrow and that even more eosinophils (about 40%) in tissue adjacent to oral carcinomas contain immunoreactive TGF-α product. Thus eosinophil-associated TGF-α may be present as preformed stores in some bone marrow eosinophils but further production of TGF-α may be induced in eosinophils infiltrating sites of certain pathologic processes. In this respect, production of TGF-α by hamster eosinophils may bear analogy to TNF-α production by mouse mast cells. Mouse mast cells constitutively contain substantial amounts of preformed TNF-α, but specific stimulation of the cells *via* the IgE receptor results in elevated levels of TNF-α mRNA and synthesis of additional product.[24] Recently it was reported that some cell types can express an active, membrane-associated form of TGF-α.[25–27] However, in the face of the extensive reactivity for TGF-α seen with hamster eosinophils, it was not possible to determine if these cells also exhibited membrane-associated staining.

Although it remains to be shown whether TGF-α expression is a characteristic common to all mammalian eosinophils, the findings reported here prompted us to investigate whether human eosinophils contain TGF-α. We found that eosinophils infiltrating the stroma of human carcinomas expressed TGF-α mRNA and product, as did the circulating eosinophils of a patient with the hypereosinophilic syndrome.[28] The identification of eosinophils as a potential source of TGF-α clearly permits speculation about novel roles for the eosinophil in health and disease. But it should be emphasized that the biologic role(s) of eosinophil-associated TGF-α remain to be determined. We have no data indicating whether the eosinophil-associated cytokine is identical to that of other cells or expresses differences in structure or bioactivity. Nevertheless the known activities of TGF-α suggest that eosinophils might influence epithelial cell proliferation. Eosinophil-dependent augmentation of epithelial cell proliferation would be particularly likely in anatomic sites where eosinophils are abundant, such as the normal intestine, or in diseases associated with large numbers of infiltrating eosinophils.

In our hamster cheek pouch model of oral carcinogenesis, both *in situ* hybridization and immunohistochemistry indicated that the hyperplastic or neoplastic squamous epithelium can supply TGF-α by an autocrine mechanism. As a result, any TGF-α provided by infiltrating eosinophils may not importantly contribute to the processes of carcinogenesis or tumor growth in this setting. On the other hand, Schreiber et al[29] demonstrated that TGF-α is a potent inducer of angiogenesis in the hamster cheek pouch. In our study, many of the TGF-α–positive eosinophils associated with the carcinomas occurred in close proximity to the tumor's vasculature. The production of TGF-α represents a novel mechanism by which eosinophils might contribute to neovascularization during tumor development and other pathologic processes.

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