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Rapid Communication

The Eosinophil as a Cellular Source of Transforming Growth Factor Alpha in Healing Cutaneous Wounds

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Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are known to promote the healing of epithelial wounds. Eosinophils are present in healing wounds and have recently been shown to be capable of producing TGF-α. This investigation was done to determine if eosinophils infiltrated into healing wounds are capable of expressing this cytokine. Using the rabbit cutaneous open wound model, the study found that the eosinophil is one of the predominant cell types in the healing wound, beginning from the seventh day and thereafter. Most surprisingly, the majority of the eosinophils present in the healing wound were found to contain TGF-α mRNA and protein by in situ hybridization and immunohistochemistry. Thus it is proposed that the delivery of TGF-α by eosinophils to epithelial wound healing sites represents a normal body mechanism whereby this multifunctional cytokine can accelerate the wound healing process. (Am J Pathol 1991, 138:1307–1313)

Exogenous application of epidermal growth factor (EGF) or transforming growth factor-alpha (TGF-α), topical or sustained release, has been shown to facilitate the healing of cutaneous wounds.[1] Recently endogenous production of EGF,[2] EGF-like substances,[3] and TGF-α[4,5] has been detected in healing wounds. Together this evidence suggests that there might be cellular sources of these cytokines at wound sites that could facilitate the healing process. Macrophages have been identified as a cellular source of TGF-α in healing wounds through a mRNA phenotyping procedure.[5] However cellular localization of TGF-α by *in situ* investigations has not been performed.

Eosinphils are known to be present in the inflammatory infiltrate associated with the healing of cutaneous wounds;[6,7] however the significance of their presence is not known. Past studies have implicated eosinophils in the modulation of collagen metabolism.[7,8] We recently demonstrated the production of TGF-α by eosinophils infiltrated into developing carcinomas in both hamsters and humans.[9,10] The aim of this study is to determine if eosinophils infiltrated into healing wounds can express TGF-α using a well-characterized rabbit cutaneous open wound model.[11]

Materials and Methods

Rabbit Cutaneous Wounds

Wounds were made in 20 New Zealand white rabbits weighing 3 to 5 kg, as previously described.[11] Anesthesia was induced using intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg). Three by three square centimeter wounds were made on the flanks of rabbits to the level of the panniculus carnosus muscle. Wounds from two animals were harvested on days 2, 7, 8, 9, 11, 13, 14, 17, and 21. Histologic quantification of eosinophils was performed on all the collected wounds, including normal rabbit skin. *In situ* hybridization and immunohistochemis-

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try were performed on day 2, 7, 8, 9, 11, 13, 21 wounds, including normal rabbit skin.

Quantification of eosinophils infiltrated into the healing wounds was performed as follows. Wound sections from the two rabbits at each of the time points were stained with the Fisher Giemsa stain (SG-28) and examined using rhodamine fluorescence at ×250. An optical grid was used to assist alignment and quantification. The grid was aligned such that one side was always parallel to the surface. The cell layers within the grid, immediately adjacent to the wound surface, were counted. Ten fields from each of the two rabbits at each time point were counted. These numbers were pooled (n = 20) and the means and standard deviation calculated.

In Situ *Hybridization*

In situ hybridization was done according to conditions that would permit specific labeling of eosinophil cytoplasmic mRNA, as previously described.[9,10,12] ^{35}S-labeled human[9] and hamster[13] TGF-α single-stranded riboprobes were used.

Immunohistochemistry

Immunohistochemical detection of TGF-α protein was performed according to procedures previously described.[9,10] 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, Manhasset, NY) was used. Sections were counter stained with 0.2% aniline blue for 10 minutes to identify eosinophils.[14]

Results

The healing of cutaneous wounds on the flanks of New Zealand white rabbits takes approximately 21 days.[11] Examination of histologic sections from different time intervals during the healing process revealed a striking profile of infiltrating eosinophils (Figure 1). Figure 1 is a montage of a day-7 wound stained with Giemsa and viewed with rhodamine fluorescence, demonstrating the large numbers of infiltrated eosinophils. The epithelial edges are indicated by the white arrows. Besides eosinophils, the keratinized layer of the epithelium also fluoresces. Each frame was taken at ×100 original magnification. Staining of histologic sections with a Giemsa stain (Fisher Scientific, SG-28) and subsequent viewing with rhodamine fluorescence allows identification of virtually all eosinophils present.[9,10] This permits quantification of eosinophils throughout the healing process (Figure 2). Normal rabbit skin contained 0.7 ± 1.0 eosinophils per field of examination (×250). On day 7 of the healing process, the number of eosinophils increased to 532 ± 175. By day 21, the number of eosinophils returned to approximately baseline level (3.2 ± 6.3). The density of eosinophils is highest within the dimension of the healing wound, increasing toward the surface (Figure 1). The eosinophils formed a continuous bridge between the injured epithelial edges. In day-7 wounds, the surface was so densely packed with eosinophils that they formed a layer of about three or four cells thick that was composed almost exclusively of eosinophils (Figure 3A). Figure 3B is a higher-power examination of the fast green-stained section (center of a day-7 wound) demonstrating the presence of eosinophils and their characteristic cytoplasmic granules.[15]

To determine if the infiltrated eosinophils synthesize TGF-α mRNA in these wound sites, *in situ* hybridization was performed. Eosinophils present in the dermis of normal rabbit were not labeled for TGF-α mRNA. However, in day-2 wounds, a large percentage (72% ± 4.6%) of infiltrated eosinophils were found to contain TGF-α mRNA. By day 7, almost all eosinophils adjacent to the wound surface were found to contain TGF-α mRNA (96.8% ± 1.6%). By day 21, when the wound had completely re-epithelialized, some eosinophils were found to contain TGF-α mRNA (20.1% ± 25.1%). The large variability of TGF-α mRNA activity in eosinophils observed in the day 21 wounds might reflect the heterogeneity of microenvironment in the wounds examined. Quantification of TGF-α mRNA-positive eosinophils in the healing wounds is shown in Figure 2. Figure 3C to F illustrates the detection of TGF-α mRNA in a day-7 wound. Figure 3C is a bright-field view of the wound labeled with a ^{35}S-antisense hamster TGF-α riboprobe. Figure 3D is the dark-field visualization of the same field using a green filter, highlighting the autoradiographic grains. Figure 3E is the visualization of the same field using rhodamine fluorescence, demonstrating the presence of many eosinophils in the wound, increasing numbers toward the surface. Figure 3F is a composite exposure using

Figure 1. *A montage to demonstrate eosinophils in a 7-day rabbit cutaneous healing wound. The section was stained with a Fisher Giemsa (SG-28) and observed using rhodamine fluorescence optics. Eosinophils fluoresce bright orange.[9,10] Notice the keratinized layer of the epithelium also fluoresces. Each frame was taken at ×100 original magnification. Each frame was color adjusted to match each other. However, due to the large number of units (43 frames in all), slight mismatches in color and/or intensities were unavoidable. The entire section was evenly stained by Giemsa; the slight difference in fluorescence intensities (for example, the right* versus *the left side) is primarily due to the color adjustment process in producing the individual frames. The white arrows indicate the epithelial edges that border the ulcerated wound.*

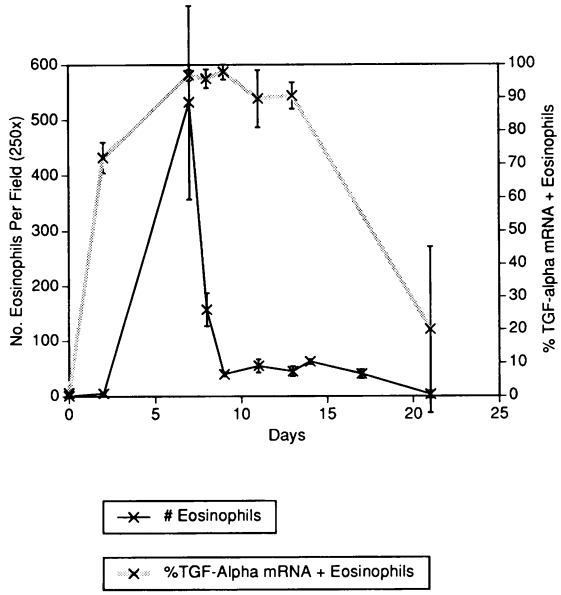


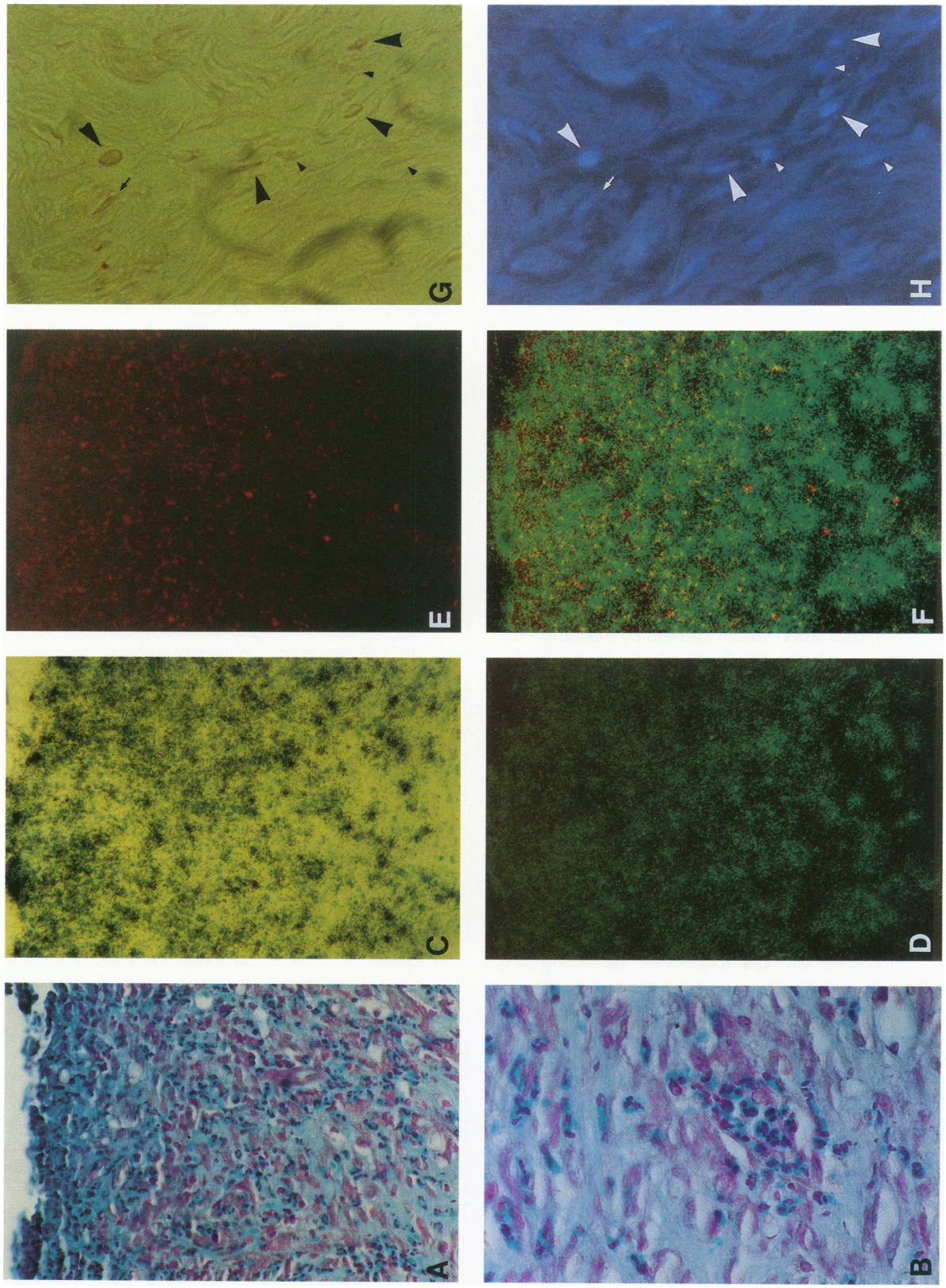
Figure 2. *Eosinophil density and TGF-α mRNA activity during cutaneous wound healing in rabbit. Quantification of eosinophils and eosinophil positive for TGF-α mRNA were performed as described (see Materials and Methods). Wounds from two animals were harvested on days 2, 7, 8, 9, 11, 13, 14, 17, and 21. Histologic quantification of eosinophils were performed on all the collected wounds, including normal rabbit skin.* In situ *hybridization was performed on day 2, 7, 8, 9, 11, 13, 21 wounds, including normal rabbit skin.*

rhodamine fluorescence followed by darkfield to demonstrate the localization of TGF-α mRNA autoradiographic grains to the fluorescent eosinophils. Higher-power examinations revealed that most of the autoradiographic grains were localized to the fluorescent eosinophils. Eosinophils in periwound areas labeled more intensely than those found within the wound proper. All wounds were labeled using sense and antisense ^{35}S-labeled human and hamster TGF-α riboprobes.[9,10,13] Both human and hamster antisense ^{35}S-riboprobes yielded identical labeling patterns. Labeling intensities were slightly higher using the hamster ^{35}S-antisense TGF-α riboprobe. Sense ^{35}S-TGF-α riboprobe from either species did not label any cells (data not shown). The percentage of TGF-α mRNA-positive eosinophils adjacent to the wound surface during the 21-day wound healing period is plotted in Figure 2.

To determine if the detected TGF-α mRNA synthesized by the infiltrated eosinophils was translated into TGF-α protein, immunohistochemistry was performed using an MAb directed against the C terminus (amino acid 34–50) of the human mature TGF-α peptide. Immunostaining of wound sections for TGF-α protein revealed evidence for both cellular and extracellular TGF-α product. Figure 3G demonstrates the immunostaining for TGF-α in a day-7 wound. Five cells in this field exhibited mild positive immunoreactivity to the TGF-α MAb (one arrow and four large arrowheads). To determine if any of the TGF-α immunoreactive cells are eosinophils, the same field was observed using ultraviolet (UV) fluorescence (365 nm). These sections were counterstained with 0.2% aniline blue and the cytoplasmic granules of eosinophils exhibit a blue fluorescence when viewed using UV fluorescence.[9,10] Seven cells in the field fluoresce (four large and three small arrowheads), four of which correspond to the TGF-α immunoreactive cells in Figure 3G (large arrowheads). The aniline blue nonfluorescent TGF-α immunoreactive cell (arrow) is morphologically consistent as a fibroblast. Mild TGF-α immunoreactivity also was detected in the extracellular spaces in the healing wounds (Figure 3G), suggesting release of the mature form of the TGF-α peptide from the infiltrated eosinophils. Immunostaining of rabbit eosinophils using the human anti–TGF-α MAb yielded intensities milder than that seen with humans or hamsters.[9,10] This could be due to differences in the C terminus of the mature TGF-α between humans and rabbits or the lower level of TGF-α protein in rabbit eosinophils. The DNA or the protein sequence to rabbit TGF-α presently is not known. Staining of adjacent sections with a control MAb against the bacterial protein β-galactosidase did not yield any staining of cells or extracellular matrix. It should be noted that Rappolee et al[5] have shown that rabbit wound fluid contains 75 ng of TGF-α/EGF per liter, supporting our immunohistochemical finding.

We found that TGF-α mRNA was detectable in the majority of eosinophils (more than 90%) associated with rabbit open cutaneous wounds from days 7 to 13. By immunohistochemistry, 50% to 55% of these eosinophils were positive for TGF-α product. These findings are sim-

Figure 3. *Demonstration of eosinophils, eosinophil-derived TGF-α mRNA, and eosinophil-derived TGF-α protein in 7-day rabbit cutaneous healing wounds.* **A:** *Ulcerated portion of a day-7 wound stained with an eosinophil-specific stain, fast green.[15] Original magnification, ×300.* **B:** *Higher-power view of fast green-positive eosinophils demonstrating morphologic details. Original magnification, ×600.* **C to F:** *Ulcerated portion of a day-7 wound hybridized to a ^{35}S-labeled antisense hamster TGF-α riboprobe. Exposure time was for 48 hours at 4°C. Original magnification, ×100.* **C:** *Bright-field visualization.* **D:** *Dark-field visualization highlighting the autoradiographic signals using a green filter.* **E:** *Fluorescence visualization using rhodamine filters demonstrating the fluorescence of human eosinophils.* **F:** *Double-exposure visualization, first with dark-field followed by rhodamine fluorescence. Similar labeling patterns were obtained with an ^{35}S-labeled antisense human TGF-α riboprobe. Labeling of adjacent sections with either human or hamster ^{35}S-labeled sense TGF-α riboprobes did not result in the labeling of any cell.* **G–H:** *Immunohistochemical detection of TGF-α protein in a day-7 rabbit wound. Original magnification, ×400.* **G:** *Stained with a human TGF-α MAb at 18 μg/ml.* **H:** *Visualization of aniline blue fluorescence of eosinophils by fluorescent microscopy at 365 nm with DAPI filter. No cell in adjacent sections stained with a control MAb (bacterial β-galactosidase). Large arrowheads: eosinophils stained mildly positive for TGF-α (red). Small arrowheads: eosinophils that did not stain for TGF-α. Arrow: nonaniline blue fluorescent cell stained for TGF-α.*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ilar to that demonstrated for human eosinophils infiltrated into colon and oral carcinomas.[9] 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 were 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 macrophates stimulated with lipopolysaccharide but not in the unstimulated cells.[16]

Discussion

This report demonstrates that eosinophils infiltrated into healing rabbit cutaneous open wounds can elaborate a cytokine, TGF-α, which has been shown to promote wound healing. Although the presence of eosinophils in cutaneous wounds have been noted previously,[7] the significance of this cell in healing wounds has not received much investigation. Our experience indicates that the identification of eosinophils in histologic sections often can be difficult, even with specific granulocyte stains such as Wright or Giemsa. We found that staining with a Fisher Giemsa stain (SG-28) followed by rhodamine fluorescence allows virtually all eosinophils to be identified. This staining and visualization procedure permits the demonstration of the striking abundance of eosinophils in healing wounds, as shown in Figures 1 and 2. It is known that a cell type, the heterophil, morphologically resembles the eosinophil in a number of species, including the rabbit. We have performed extensive histochemical staining of these sections with fast green,[15] aniline blue,[14] Giemsa,[9,10] and hematoxylin and eosin,[17] examined using bright-field and appropriate fluorescent optics, and concluded that the TGF-α–positive cells in the rabbit open cutaneous wounds to be consistent as eosinophils.

The ability of eosinophils to infiltrate into wound sites and produce TGF-α allows speculation that this cell might deliver optimal local concentrations of this cytokine to facilitate healing. The known biologic activities of TGF-α, including mitogenicity,[18] angiogenicity,[18] promotion of keratinocyte migration,[19] and autoinduction of TGF-α expression in keratinocytes,[20] make it an important cytokine to be present in healing cutaneous wounds. Topical application of TGF-α has been shown to promote epithelial wound healing.[21] Transforming growth factor-alpha was found to be present in skin wounds of mice[5] and in regenerating gastric mucosa.[4] Furthermore wound fluid from the rabbit was found to contain significant TGF-α immunoreactivity,[5] thus corroborating our present finding. It is important to note that cell types other than eosinophils were detectable for TGF-α mRNA and protein. These include keratinocytes and other inflammatory cell types within the wound. Our analysis, however, indicates that most of the detectable TGF-α mRNA and protein were of eosinophil origin.

Our finding further supports the rationale for the exogenous application of TGF-α to wound sites in situations in which the immune response of the host is comprised, such as in patients on chemotherapy or medications that can affect the maturation, development, and activities of eosinophils. However, because the normal host usually can mount an adequate healing response, the possible benefits of additional TGF-α remains to be investigated. We propose that TGF-α derived from eosinophil in the healing wound is involved in the induction of epithelial migration, angiogenesis, and organization of the wound.

References

1. Carpenter G, Cohen S: Epidermal growth factor. Ann Rev Biochem 1979, 48:193–216
2. Wright NA, Pike C, Elia G: Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. Nature 1990, 343:82–85
3. Grotendorst GR, Soma Y, Takehara K, Charette M: EGF and TGF-alpha are potent chemoattractants for endothelial cells and EGF-like peptides are present at sites of tissue regeneration. J Cell Physiol 1989, 139:617–623
4. Beauchamp RD, Barnard JA, McCutchen CM, Cherner JA, Coffey RJJ: Localization of transforming growth factor alpha and its receptor in gastric mucosal cells. Implications for a regulatory role in acid secretion and mucosal renewal. J Clin Invest 1989, 84:1017–1023
5. Rappolee DA, Mark D, Banda MJ, Werb Z: Wound macrophages express TGF-α and other growth factors in vivo: Analysis by mRNA phenotyping. Science 1988, 241:708–712
6. Baker JR, Bassett EG, Souza P: Eosinophils in healing dermal wounds. J Anat 1976, 121:401 (Abstr)
7. Basset EG, Baker JR, De Souza P: A light microscopical study of healing incised dermal wounds in rats, with special reference to eosinophil leucocytes and to the collagenous fibers of the periwound areas. Br J Exp Path 1977, 58:581–605
8. Hibbs MS, Mainardi CL, Kang AH: Type specific collagen degradation by eosinophils. Biochem J 1982, 207:621–624
9. Wong DTW, Weller PF, Galli SJ, Elovic A, Rand TH, Gallagher GT, Chiang T, Chou MY, Matossian K, McBride J, Todd R: Human eosinophils express transforming growth factor-alpha. J Exp Med 1990, 172:673–681
10. Elovic A, Galli SJ, Weller PF, Chang ALC, Chiang T, Chou MY, Donoff B, Gallagher GT, Matossian K, McBride J, Tsai M, Todd R, Wong DTW: Production of transforming growth factor-alpha by hamster eosinophils. Am J Path 1990, 137:1425–1434

11. Bertolami CN, Donoff RB: Identification, characterization, and partial purification of mammalian skin wound hyaluronidase. J Invest Dermatol 1982, 79:417–421
12. Wong DTW, Chou MY, Chang L-C, Gallagher GT: Use of intracellular H3 mRNA as a marker to determine the proliferation pattern of normal and DMBA-transformed hamster oral epithelium. Cancer Res 1990, 50:5107–5111
13. Chiang T, McBride J, Chou MY, Nishimura I, Wong DTW: Molecular cloning of the complementary DNA encoding for the hamster TGF-α mature peptide. Carcinogenesis 1991, 12:529–532
14. McCrone EL, Lucey DR, Weller PF: Fluorescent staining for leukocyte chemotaxis. Eosinophil-specific fluorescence with aniline blue. J Immunol Methods 1988, 114:79–88
15. Ottesen EA, Cohen SG: The Eosinophil, Eosinophilia, and Eosinophil-Related Disorders. St. Louis, CV Mosby, 1978, pp 622-624
16. Madtes DK, Raines EW, Sakariassen KS, Assoian RK, Sporn MB, Bell GI, Ross R: Induction of transforming growth factor-α in activated human alveolar macrophages. Cell 1988, 53:285–293
17. Shanklin WM, Sheppard LB, Burke GWJ: Staining of rabbit eosinophils and pseudoeosinophil leukocytes. Acta Anat 1977, 97:147–150
18. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α: A more potent angiogenic mediator than epidermal growth factor. Science 1986, 232:1250–1253
19. Barrandon Y, Green H: Cell migration is essential for sustained growth of keratinocytes colonies: The role of transforming growth factor-α and epidermal growth factor. Cell 1987, 50:1131–1137
20. Coffey RJJ, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR: Production and auto-induction of transforming growth factor-α in human keratinocytes. Nature 1987, 328:817–820
21. Schultz GS, White M, Mitchell R, Brown G, Lynch J, Twardzik DR, Todaro GJ: Epithelial wound healing enhanced by transforming growth factor-alpha and vaccinia growth factor. Science 1987, 235:350–352