

DETECTION OF TRANSFORMING GROWTH FACTOR α IN
NORMAL, MALIGNANT, AND HYPERPROLIFERATIVE
HUMAN KERATINOCYTES

ALICE B. GOTTLIEB,* CHEOW KHONG CHANG,‡ DAVID N. POSNETT[§]
B. FANELLI,* AND JAMES P. TAM‡

*From the *Laboratories of Immunology and Investigative Dermatology, ‡Department of Biochemistry, The Rockefeller University; and the [§]Department of Medicine, Cornell University Medical College, New York, New York 10021*

Transforming growth factor α (TGF- α) is a 50-amino acid peptide that is structurally homologous to vaccinia virus growth factor and epidermal growth factor (EGF) (1). TGF- α exerts its biologic effects by binding to the EGF receptor (reviewed in reference 2). It was initially described only in virally transformed cell lines and in a variety of human tumors, and was demonstrated to cause transformation in vitro (2-4). However, TGF- α has been shown to inhibit gastric acid secretion, promote angiogenesis, cause calcium resorption from bone, influence estrogen metabolism, and promote wound healing in normal tissues (reviewed in reference 2). In this report, TGF- α expression in skin from normal individuals, from patients with psoriasis, and patients with malignant skin diseases will be demonstrated using an mAb raised against synthetic human TGF- α .

Materials and Methods

Production and Characterization of mAbs. mAbs were produced against synthetic human TGF- α (1) using standard methodology (5). Positive hybridoma clones were subcloned four times by the limiting dilution technique. Epitope mapping of mAb A1.5 by synthetic peptides derived from TGF- α (1) was performed using an ELISA with the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). EGF was purchased from Collaborative Research, Inc. (Bedford, MA).

Immunoperoxidase Staining. Immunoperoxidase studies of formalin-fixed frozen skin sections and cytocentrifuged keratinocyte suspensions was performed using the Vectastain ABC immunoperoxidase staining system (6). Human keratinocytes obtained from either neonatal foreskin or adult breast skin were cultured in serum-free MCDB 153 medium (5, 7). For competition experiments, undiluted mAb A1.5 supernatant was mixed with purified synthetic human TGF- α (2 μ g/ml) immediately before incubation with the skin sections. Staining was compared with that of the same antibody mixed with an equal volume of PBS just before addition.

Results

Characterization of mAb. From one fusion with splenocytes of 2 mice, 30 positive clones were generated. Three clones, A1.5, A9.10, and B6.1, exhibited

This work was supported in part by grants AR-35676, AI-19080, CA-42046, CA-42073-02A1, and CA-36544 from the U.S. Public Health Service; by General Clinical Research Center grant RR-00102 from the National Institutes of Health to The Rockefeller University Hospital; and by the Pew Trusts and Triton Biosciences, Inc.

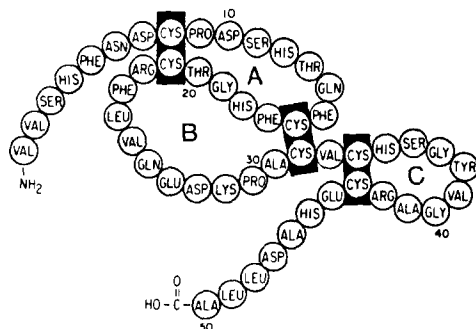


FIGURE 1. Structure of human TGF- α . The three synthetic peptide loops used in the antigenic specificity studies are indicated by A, B, and C.

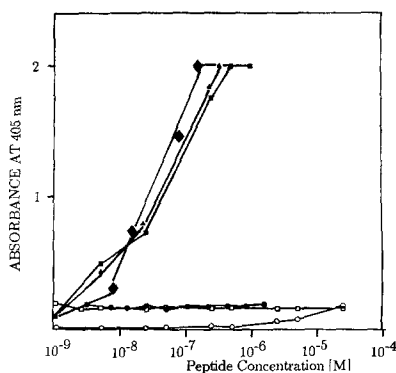


FIGURE 2. Epitope mapping of mAb A1.5 by synthetic peptides in ELISA. TGF- α (\blacklozenge), EGF (\bullet), BC-loop (\blacksquare), B-loop (\blacktriangle), A-loop (\circ), and C (\square).

the highest titers and were chosen for further study. All were of the IgG1 isotype. To determine the epitopes recognized by these mAbs, homogeneous synthetic peptides covering the whole TGF- α molecule were used (Fig. 1). TGF- α is a tricyclic protein with three disulfide loops. Loops A and B form a fused ring whereas loop C forms an independent disulfide ring. Loop C represents the segment with the strongest homology with EGF, with 6 of 10 residues being conserved (Cys_x₂GlyTyrxGlyxArgCys; where x represents other amino acids). In contrast, loop B represents the segment with the least homology with EGF, with only 4 of 17 residues being conserved (Cys_x₂GlyxCys_x₁₀cys) (1). In a sensitive ELISA, mAb A1.5 bound to loop B but not loops A or C even at peptide concentrations of 0.1 mg/ml (Fig. 2).

TGF- α is expressed by normal and malignant keratinocytes. Formalin-fixed frozen skin sections were tested for reactivity with mAb A1.5 using the immunoperoxidase technique (Fig. 3). Keratinocytes in eight nodular basal cell carcinomas (*a* and *b*), one morpheic basal cell carcinoma (*d*), and one squamous cell carcinoma (*e*) demonstrated intense staining with mAb A1.5. Of even greater interest was the observation that the overlying normal epidermis (*c*), as well as the epidermis from five normal skin specimens, were stained by the mAb. The pattern of staining was membranous. Keratinocytes in the epidermal appendages, which are developmentally derived from basal keratinocytes, were also reactive with mAb A1.5. mAb A9.10 showed an identical staining pattern, as did mAb A1.5. mAb B6.1 as well as an isotype control were nonreactive with both normal and malignant keratinocytes. Keratinocytes in plaques from 18 psoriasis patients were reactive with mAb A1.5 (Fig. 3*f*). In general, they were more intensely

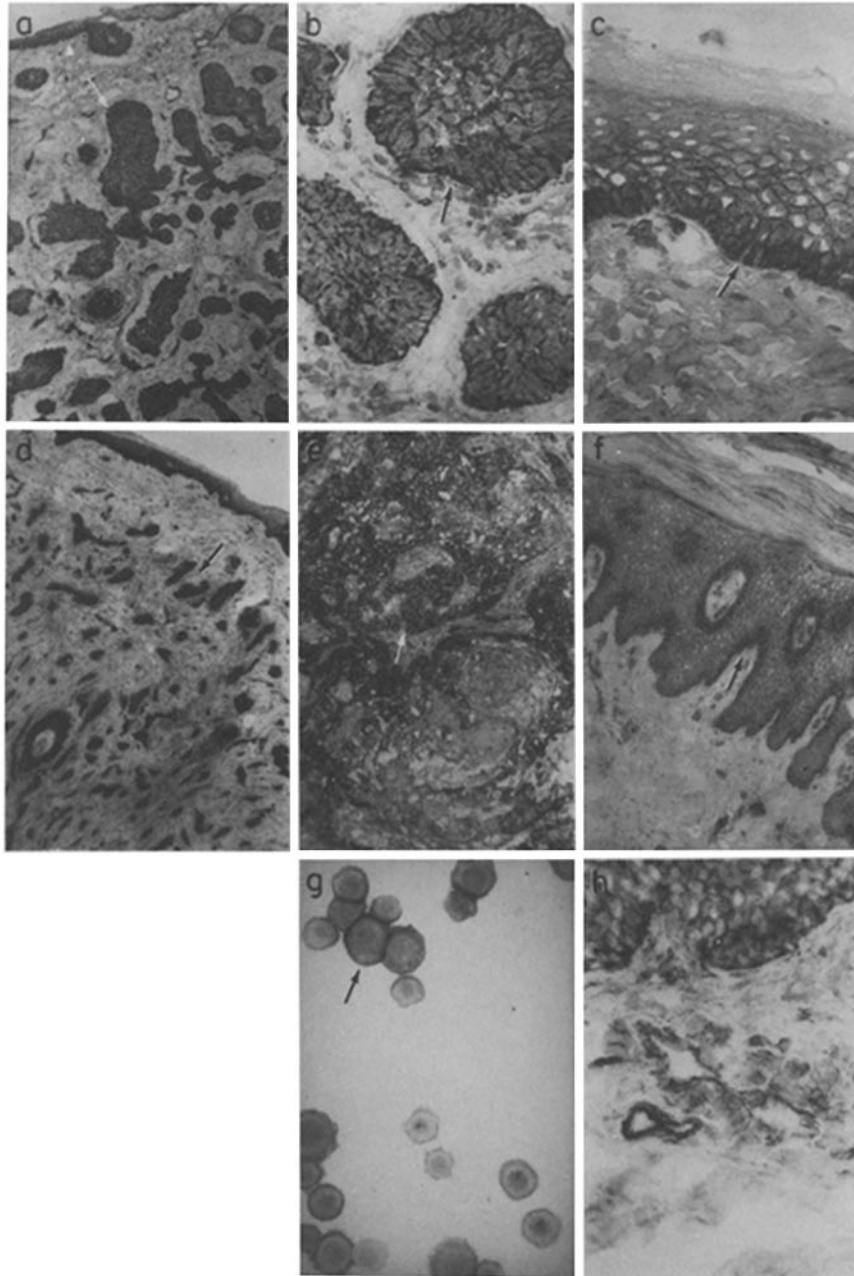


FIGURE 3. Immunoperoxidase reactivity of human skin with the anti-TGF- α mAb, A1.5. $\times 150$. Keratinocytes in nodular (*a* and *b*, $\times 615$) and morpheic (*d*) basal cell carcinomas, and in a squamous cell carcinoma (*e*) demonstrated intense staining (*black* and *white* arrows). The overlying epidermis (*a*, *arrowhead*; *d*), as well as the epidermis from normal skin (*c*, *arrow*) and psoriatic plaques (*f*, *arrow*) were also stained by the mAb. The mononuclear dermal infiltrates of both psoriasis (*f* and *h*, $\times 623$) and basal cell carcinoma specimens (*a* and *b*) showed reactivity with mAb A1.5. In addition, endothelial cells were reactive (*h*). Cultured normal keratinocytes demonstrated clear membrane reactivity with mAb A1.5 (*g*, *arrow*).

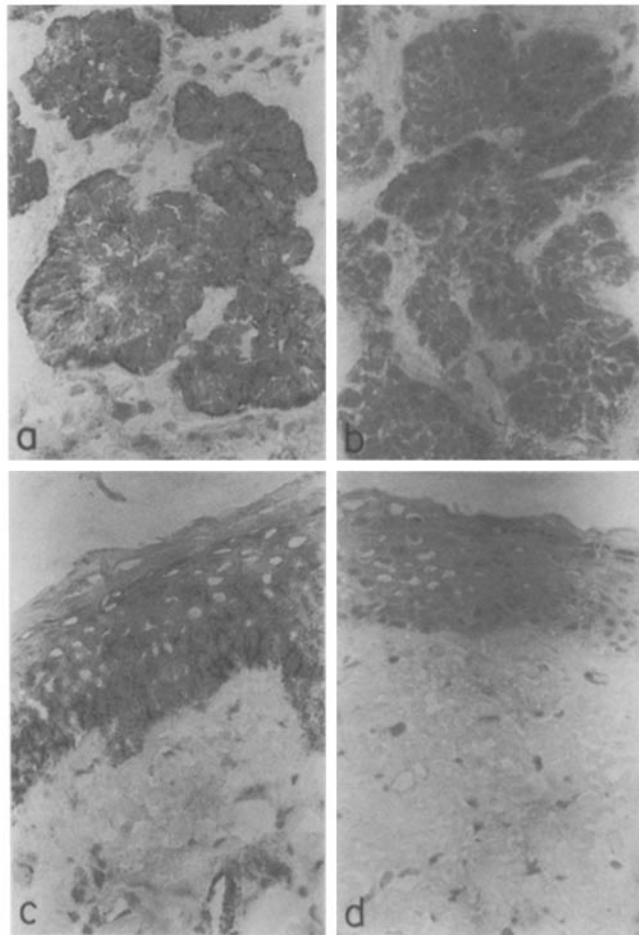


FIGURE 4. TGF- α competitively inhibits binding of mAb A1.5 to skin. $\times 713$. Synthetic human TGF- α ($2 \mu\text{g}/\text{ml}$), when added to mAb A1.5 immediately before the mixture was incubated with basal cell carcinoma (b) and normal (d) skin sections, completely removed the immunoperoxidase staining of mAb A1.5. Sham-treated mAb A1.5 showed reactivity with the same basal cell carcinoma (a) and normal skin (c) sections.

stained than those from normal skin. Cultured normal keratinocytes isolated from breast skin were trypsinized and then cytocentrifuged onto glass slides. Clear membrane staining with the anti-TGF- α antibody was observed (Fig. 3g).

In the mononuclear dermal infiltrates of both psoriasis and basal cell carcinoma specimens there was clear reactivity with mAb A1.5 (Fig. 3h). We have previously demonstrated that these infiltrates contained activated T cells, macrophages, and Langerhans' cells (6). In the psoriatic plaques, endothelial cells also appeared to be stained. The exact identity of the cell(s) reacting with the anti-TGF- α antibody is still under active investigation.

To confirm that the reactivity of normal and malignant keratinocytes with the anti-TGF- α antibodies was specific, competition experiments were performed. Synthetic human TGF- α , at a concentration of $2 \mu\text{g}/\text{ml}$, was added to mAb A1.5 immediately before the mixture was incubated with basal cell carcinoma and normal skin sections. TGF- α completely removed the reactivity of mAb A1.5 with both the basal cell tumor and normal epidermis (Fig. 4). EGF at similar concentrations did not compete in binding to mAb A1.5.

Discussion

TGF- α was demonstrated on the membrane of normal, hyperproliferative, and malignant keratinocytes. Its presence in normal keratinocytes strongly suggests that it plays an important role in the normal physiological control of keratinocyte proliferation. Inhibition of TGF- α -induced transformation may be effected by local cellular and humoral elements as has been described for the viral *myc* and *ras* oncogenes (8).

The presence of TGF- α in keratinocytes and activated platelets (9), along with its known mitogenic effect, suggests that it may play a significant role in wound healing. In laboratory animals, TGF- α has been used successfully in treating experimentally induced wounds (10). TGF- α and EGF are structurally homologous to fibronectins, proteoglycans, blood clotting factors IX and X and protein C (1). These proteins play an important role in the body's response to wounding and in tissue repair.

Previous studies have focussed on the expression of TGF- α by transformed cells (2-4). These studies failed to detect TGF- α in normal cultured fibroblasts, hematopoietic cell lines, and whole liver. However, TGF- α has been detected in ascites from patients with cirrhosis, breast, activated platelets, and rodent fetuses (reviewed in reference 2). At the completion of this manuscript, Coffey et al. (11) reported the production of TGF- α in keratinocytes.

In our studies, it was apparent that TGF- α was present on cells in the dermal infiltrates of inflammatory, hyperproliferative (psoriasis), and neoplastic skin diseases. In addition, endothelial cells from psoriatic plaques appeared to contain TGF- α . Double-labeling immunoperoxidase experiments are in progress to identify whether macrophages, Langerhans' cells, and/or activated T cells contain TGF- α . These cells are found in the dermal infiltrates of delayed type hypersensitivity reactions (12, 13) and of inflammatory and hyperproliferative skin diseases such as psoriasis and lichen planus (6). TGF- α and possibly other factors that promote keratinocyte proliferation may play a significant role in the generation of the acanthosis seen in these conditions.

Summary

Transforming growth factor α (TGF- α) is a 50-amino acid peptide, previously demonstrated only in transformed cell lines and human tumors, which is structurally homologous to epidermal growth factor (EGF). TGF- α expression in keratinocytes from normal individuals, patients with psoriasis, and patients with malignant skin diseases was investigated using an mAb raised against synthetic human TGF- α .

mAb A1.5 reacted with TGF- α , but not EGF, in a sensitive ELISA. Keratinocytes in eight nodular basal cell carcinomas, one morpheic basal cell carcinoma, and one squamous cell carcinoma demonstrated intense membranous immunoperoxidase staining with mAb A1.5. Of even greater interest was the observation that the overlying normal epidermis, as well as the epidermis from five normal skin specimens, were stained by the mAb. Keratinocytes in plaques from 18 psoriasis patients were more intensely stained than those from normal skin. Cultured normal keratinocytes demonstrated membranous staining with mAb A1.5. Absorption of mAb A1.5 with synthetic human TGF- α completely re-

moved the reactivity of mAb A1.5 with both basal cell tumors and normal epidermis.

The demonstration of TGF- α in normal keratinocytes suggests that it plays a role in normal keratinocyte growth, wound healing, and in the pathogenesis of acanthosis.

We thank Dr. Philip Prioleau and the members of the Division of Dermatology at The New York Hospital and of The Rockefeller University Hospital for their help in these studies. We also thank Ms. Cathy Volin for her excellent technical assistance.

Received for publication 30 September 1987 and in revised form 23 November 1987.

References

1. Tam, J. P., M. A. Sheikh, D. S. Solomon, and L. Ossowski. 1986. Efficient synthesis of human type alpha transforming growth factor: its physical and biological characterization. *Proc. Natl. Acad. Sci. USA.* 83:8082.
2. Derynck, R. 1986. Transforming growth factor-alpha: structure and biological activities. *J. Cell. Biochem.* 32:293.
3. Anzano, M. A., A. B. Roberts, C. A. Meyers, A. Komoriya, L. C. Lamb, J. M. Smith, and M. B. Sporn. 1982. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. *Cancer Res.* 42:4776.
4. DeLarco, J. E., and G. J. Todaro. 1978. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA.* 75:4001.
5. Gottlieb, A. B., D. N. Posnett, M. K. Crow, T. Horikoshi, L. Mayer, and D. M. Carter. 1985. Purification and in vitro growth of human epidermal basal keratinocytes using a monoclonal antibody. *J. Invest. Dermatol.* 85:299.
6. Gottlieb, A. B., B. Lifshitz, S. M. Fu, L. Staiano-Coico, C. Y. Wang, and D. M. Carter. 1986. Expression of HLA-DR molecules by keratinocytes and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. *J. Exp. Med.* 164:1013.
7. Peehl, D., and R. G. Ham. 1980. Clonal growth of human keratinocytes with small amounts of dialyzed serum. *In Vitro.* 16:526.
8. Newmark, P. 1987. Oncogenes and cell growth. *Nature (Lond.).* 327:101.
9. Sporn, M. B., and A. B. Roberts. 1986. Peptide growth factors and inflammation, tissue repair, and cancer. *J. Clin. Invest.* 78:329.
10. Schultz, G. S., M. White, R. Mitchell, G. Brown, J. Lynch, D. R. Twardzik, and G. J. Todaro. 1987. Epithelial wound healing enhanced by transforming growth factor-alpha and vaccinia growth factor. *Science (Wash. DC).* 235:350.
11. Coffey, R. J., Jr., R. Derynck, J. N. Wilcox, T. S. Bringman, A. S. Goustin, H. L. Moses, and M. R. Pittelkow. 1987. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature (Lond.).* 328:817.
12. Kaplan, G., M. D. Witmer, I. Nath, R. M. Steinman, S. Laal, H. K. Prasad, E. N. Sarno, U. Elvers, and Z. A. Cohn. 1986. Influence of delayed immune reactions on human epidermal keratinocytes. *Proc. Natl. Acad. Sci. USA.* 183:3469.
13. Kaplan, G., A. Nusrat, M. D. Witmer, I. Nath, and Z. A. Cohn. 1987. Distribution and turnover of Langerhans cells during delayed immune responses in human skin. *J. Exp. Med.* 165:763.