Cytokine mRNA Expression in Normal Skin of Various Age Populations Before and After Engraftment onto Nude Mice

AMOS GILHAR[1], YEHUDA ULLMANN[1], RAYA SHALAGINO[1] and GARY WEISINGER [2]

[1]Skin Research Laboratory and [2]Department of Clinical Biochemistry, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel.

Whether the impact of skin biological age on cytokine expression is a result of this tissue's proliferation potential or not is an important issue in dermatology. We investigated these questions by monitoring cytokine marker mRNA expression from human skin samples from healthy groups of individuals. The skin samples studied represented three age groups: fetal (17–21 weeks), young (18–35 years) and aged (76–88 years).

Furthermore, upon skin transplantation of tissue from different age groups onto nude mice, we investigated whether cytokine marker RNA levels would change or normalize. Interestingly, both TNF-alpha and P53 mRNA showed a similar pattern of expression. Both were significantly higher in fetal skin ($p < 0.0001$ and $p < 0.05$, respectively), and no difference was noted between aged versus young skin. In contrast to this, IL1-alpha mRNA was expressed at its lowest and highest levels in fetal and young skin, respectively. Following skin transplantation, cytokines and P53 mRNA expression were normalized to similar levels in all age groups. This study implies that when cytokine expression was determined directly at the mRNA level, post-natal expression was not significantly different at either age group. Furthermore, it seems that the environmental conditions surrounding the grafted human skin found on nude mice encouraged normalization of donor cytokine expression.

Human senescence is characterized by a variety of immunological changes. Certain immunological parameters (1–3), including delayed allograft rejection (4), depressed humoral immunity (5), diminished responsiveness in the mixed lymphocyte reaction (6) and decreased interleukin-2 production (7), have been documented in the aged population. Other aspects of the skin immune system are also altered in the elderly (8, 9). We have recently shown that while intradermal injections of gamma interferon (IFN-gamma) in young volunteers enhanced the induction of HLA-DR and ICAM-1 by epidermal cells, no effect was observed in the elderly (10, 11). This may be related to the known decrease of epidermally derived thymocyte-activating factors, including an "IL-1-like" cytokine, with age (12). However, the etiology of the various age-related changes observed in immunological function is not yet well defined. Recently, we showed complete normalization of histological parameters of aged and fetal skin following engraftment onto nude mice (13). Additionally, aged skin is considered to be less responsive to cytokine stimulation (12, 14). Moreover, it has also been suggested that aged skin secretes fewer cytokines compared to young skin (15). The present investigation was therefore undertaken to analyse the impact of biologic age on the expression of the skin tumor necrosis factor alpha (TNF-alpha), interleukin 1-alpha (IL1-alpha), interleukin 6 (IL6) and the transcription factor, P-53 mRNA levels. Furthermore, we also studied whether skin mRNA levels of these factors can be changed or normalized by grafting skin onto nude mice.

MATERIALS AND METHODS

Human skin samples

Seven elderly volunteers (3 men and 3 women), aged 76 to 88 years (mean age 82 ± 4) and known to be in good health, and 7 young healthy volunteers (6 men and one woman), aged 18 to 35 years (mean 31 ± 9), were recruited for this study after giving informed consent. From each subject 0.4-mm split-thickness skin grafts were obtained, using a dermatome knife. We used this thickness to essentially isolate the skin epidermis. The skin was obtained for engraftment onto nude mice, and Northern blot analysis was performed prior to and following skin engraftment. Human fetal skin was obtained from the trunks of aborted fetuses (4 males and one female) with estimated gestational ages of 17–21 weeks, mean 19.6 ± 1.5. All skin samples were dissected from the underlying soft tissue, released into RPMI medium for 30 min and then transplanted onto the nude mice. All grafts of the fetal, young and aged skin sections ranged from 1 to 1.5 cm in diameter.

Skin transplantation onto and care of nude mice

The split-thickness graft was transplanted onto the subcutaneous tissue over the lateral thoracic cage of the mouse, as previously described (16). The animals used for this study were 2–3 months old Balb/C nude mice, obtained from the pathogen-free animal facility of the Faculty of Medicine, Technion-Israel Institute of Technology, Haifa.

Total RNA preparation and Northern analysis

Following biopsy, skin was snap-frozen and stored at $-80°C$ until use.

Total RNA was extracted from the tissue, using a minor modification of the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski & Sacchi, and quantified by optical density (17). Ten-microgram samples of total RNAs were electrophoresed on a denaturating glyoxal gel, transferred by the capillary transfer method in $20 \times SSC$ ($1 \times SSC = 3M$ sodium chloride, 0.39M sodium citrate) overnight to Nytran membranes (Schleicher & Schuell) and baked at 80°C for 2 h, prior to hybridization (8).

Prehybridization was performed overnight at 45°C ($5 \times$ Denhardts, $5 \times SSC$, 50 mM sodium phosphate, pH 6.5; 1% SDS, 100 µg/ml sheared salmon sperm DNA and 50% formamide) (18).

The hybridization solution was the same except for the addition of radiolabeled probe DNA and the addition of 5% dextran sulfate (18). Each blot was hybridized at 45°C to a radiolabeled ($> 10^6$ cpm/ml) double-stranded coding region probe fragment (EcoR1; 1200 bp) of pIL6 cDNA (kindly provided by Prof. Leo Sachs, Weizmann Institute of Science, Rehovot, Israel) for 24 h. This fragment was radiolabeled with [^{32}P]-dCTP using a random primer labeling kit (Primer It 11; Stratagene, La Jolla, CA).

Blots were rehybridized to a radiolabeled Hind III-Bam H1 650 bp fragment of pIL1-alpha; Eco R1 1200 bp fragment of P53 (both kindly provided by Prof. Moshe Oren, WIS, Rehovot, Israel), a 750 bp Hpa1-Hind III fragment of TNF-alpha (a gift of Prof. David Wallach,

WIS, Rehovot, Israel) and a 1600 bp Pst1 fragment of the constitutively expressed glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as an RNA loading control (19). It should be noted that each of these probes will hybridize with both human and mouse versions of their respective mRNAs under these hybridization conditions. Following each hybridization, the blots were washed twice (60°C, 0.2 × SSC/0.1% SDS for 30 min) and autoradiographed at −80°C and the films were scanned (see below) or quantified by a phosphoimager. The image intensities of exposed films were quantified by a two-dimensional scanning densitometer (HP Deskscanner) and analysed by Quantiscan software (version1.1; Biosoft, UK). Multiple exposures of each Northern blot autoradiogram were analysed, so that each point used was not saturating.

Statistics

All samples were subjected to ANOVA and to Neuman-Keuls post-hoc test analysis, where appropriate. Data represents the mean ± SEM of replicates derived from more than 4 skin samples/point.

RESULTS

TNF-alpha is known to be a proinflammatory cytokine, leading to the release of various other cytokines from the skin (20–22). To examine the expression of TNF-alpha mRNA during skin a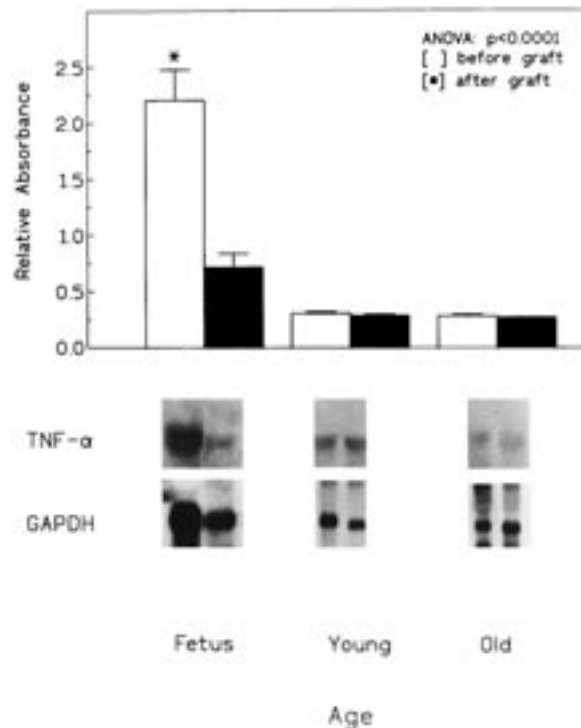ging and transplantation Northern blot analysis of human skin steady state total RNA was initiated (Fig. 1). These skin samples represented three age groups: fetal, young and aged, both before and after transplantation onto nude mice. TNF-alpha RNA expression was always significantly greater ($p < 0.0001$, Neuman-Keuls post-hoc test) from skin derived from human fetus (Fig. 1). We observed no difference between skin samples from young versus aged individuals. Although following transplantation no differences in TNF mRNA levels were observed in the young and aged groups, a dramatic and significant decrease was observed in the fetal group.

Another proinflammatory cytokine, IL1-alpha (23), was also examined in the context of our model system (Fig. 2). In contrast to our TNF-alpha data, IL1-alpha was expressed at its lowest and highest level in fetal and young skin, respectively. Interestingly, IL1-alpha mRNA levels were expressed at intermediate levels (with respect to fetal and young) in aged skin. Following transplantation onto nude mice, IL-1 levels were normalized to similar intermediate levels from all age groups.

IL6 is a cytokine known for its role in regulating epidermal proliferation (15). In the current study we found similar levels of IL6 mRNA expression from all age groups, but there was a trend toward slightly lower levels in fetal skin (Fig. 3). Following transplantation of skin, IL6 levels dropped significantly ($p < 0.05$, Neuman-Keuls post-hoc test) for the young age group only. A non-statistically significant drop was also
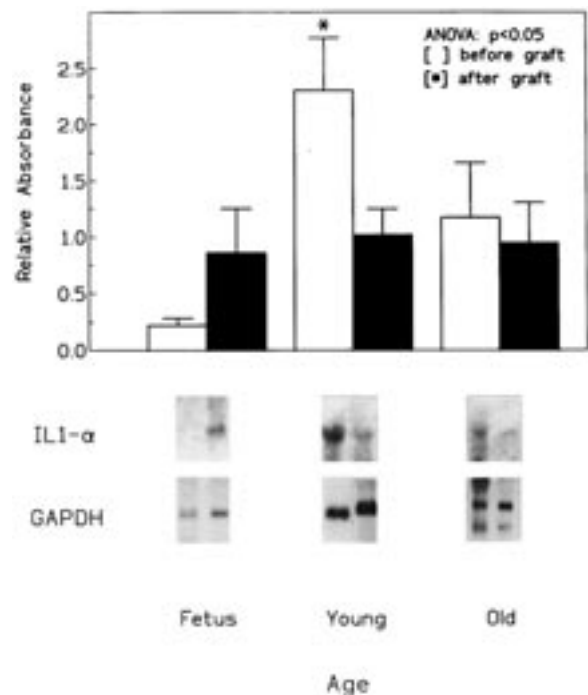

Fig. 1. Tumor necrosis factor-alpha (TNF-alpha) RNA expression in human fetal, young and old skin before and after grafting onto nude mice. Northern blot analysis was performed on total skin RNA derived from three different age groups, fresh or following transplantation onto nude mice. Each result in the histogram represents the mean ± SEM of more than four different skin samples, normalized for RNA loading to main glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA band (19). Fetal pregraft TNF-alpha mRNA expression was significantly elevated compared to all other groups ($p < 0.0001$, Neuman-Keuls post-hoc test).

Fig. 2. Interleukin 1-alpha (IL1-alpha) mRNA expression in human fetal, young and old skin before and after grafting onto nude mice. Northern blot analysis was performed as described for Fig. 1. Each result in the histogram represents the mean ± SEM of more than four different skin samples, normalized for RNA loading to the main GAPDH band (19). In this case fetal pregraft IL1-alpha mRNA expression was significantly lower than young pregraft levels ($p < 0.05$).
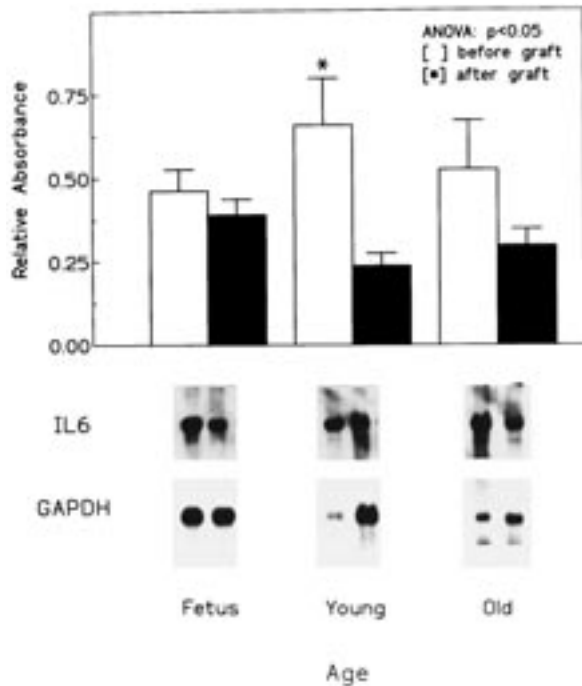
Fig. 3. Interleukin 6 (IL6) mRNA expression in human fetal, young and old skin before and after grafting onto nude mice. Northern blot analysis and data presentation are as described in Fig. 1. There was no significant difference between pregraft mRNA levels in the three age groups. There was a significant reduction of IL6 mRNA levels for young skin following engraftment ($p < 0.05$).

Fig. 4. P53 mRNA expression in human fetal, young and old skin before and after grafting onto nude mice. Northen blot analysis and data presentation are as described in Fig. 1. Fetal pregraft P53 mRNA levels were significantly higher for all other groups ($p < 0.05$).

noticed for the aged group. Variability in the aged group may account for the non-achievement of statistical significance.

P53 is expressed in normal as well as pathogenic hyperproliferating cells (24–26). Moreover, it appears that P53 is involved in the regulation of cell proliferation (26). As these three age populations (fetal, young and old) may represent different stages of skin cell proliferation, we examined their expression of P53 mRNA (Fig. 4). We found an expression profile for P53 mRNA that is remarkably similar to that of TNF-alpha (compare Fig. 1 to Fig. 4). This similarity is also obvious when comparing expression following the skin transplantation.

DISCUSSION

Many studies have shown that the younger a cellular population, the greater its proliferation potential (15, 27–30). How this proliferation potential is regulated by cytokines is still an open question.

The current dogma indicates that cytokine protein expression is reduced with aging (10–15, 27–34). In the present study, we observed no significant difference in IL6, P53 and TNF-alpha mRNA expression between young and aged populations. Differences in the translational versus transcriptional regulation for each of these three gene products may explain these apparent incongruities between their protein and RNA expression. This needs to be further studied before conclusions can be reached. Another example of a gene whose expression is not changed by aging is TGF-beta (35). An interesting
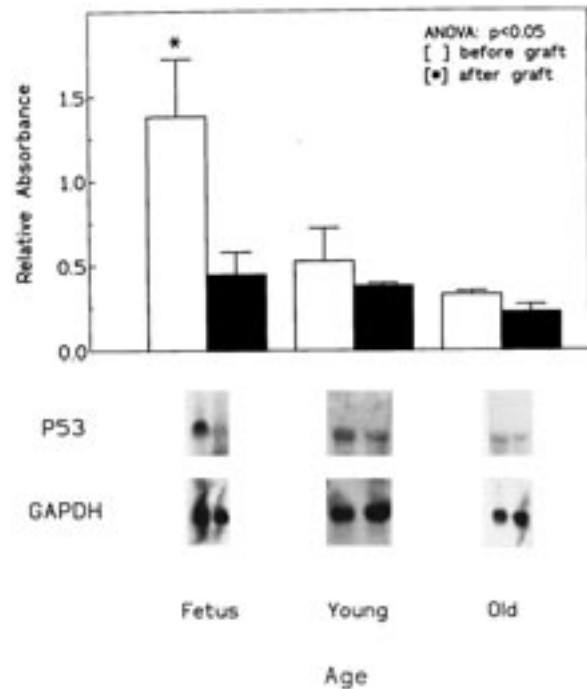
addendum to this notion is that P53 gene expression, which is normally tied to cell proliferation (24–26), was not found to be expressed differently in young and old skin samples, thus suggesting that cell proliferation levels were not very different in these two populations of skin samples. In the current study, we also demonstrate an example of a gene (IL1-alpha) that is expressed differently in young and old populations.

A normal hyperproliferative condition is evident in the developing fetus. In this case we expected to find an increase, not just in the P53 expression, but also in some of the other cytokines. We clearly found significantly elevated P53 and TNF-alpha mRNA expression in fetal skin as compared to other age groups. In contrast to this, both IL6 and IL1-alpha did not have elevated mRNA levels in the fetal skin. In particular, fetal IL1-alpha mRNA levels were very low when compared to all other age groups.

It is noteworthy that the detection of cytokine mRNA expression in fetal skin has not been previously reported. During fetal development, skin undergoes cell proliferation, but also cellular differentiation (13, 36). Hence, it is not unreasonable to suggest that different cytokines or cellular markers will be differently regulated prior to birth. It may be that some markers may show a specific expression profile for hyperproliferative skin cell populations.

Recently we have reported that there is marked acceleration of fetal skin maturation and restoration of aged skin following transplantation onto nude mice (13, 36). This may indicate that host skin cytokine environment may play a role in regulating graft cytokine gene expression. In the current study we found that the act of transplantation apparently normalizes

gene expression for each of the groups. This further supports our notion that host environmental factors, including host cytokines, influence the grafted skin. Other studies did not find such universal normalization of cytokine protein expression upon transplantation onto nude mice (15, 37). These differences may be a result of differences in cytokine transcriptional versus translational gene regulation.

This study implies that when the cytokine expression was determined directly at the mRNA level, post-natal expression was not significantly different. The consequence of this is that differences observed in cytokine and P53 protein expression between some aged populations may be due to translational regulatory mechanisms. Future mechanistic studies should take this into account. Furthermore, the environmental conditions of the grafted human skin found on nude mice encourage normalization of donor cytokine expression.

ACKNOWLEDGEMENT

REFERENCES

1. Ford PM. The immunology of ageing. Clin Rheum Dis 1986; 12: 1–10.
2. Horan HA, Fox RA. Ageing and the immune response – a unifying hypothesis. Mech Ageing Dev 1984; 26: 165–181.
3. Makinodan T, Kay MB. Age influence on the immune system. Adv Immunol 1980; 29: 287–330.
4. Menon M, Jaroslov BN, Koesterer R. The decline of cell-mediated immunity in ageing mice. J Gerontol 1974; 29: 499–505.
5. Heidrick ML, Makinodan T. Presence of impairment of humoral immunity in non-adherent spleen cells of old mice. J Immunol 1973; 111: 1502–1506.
6. Konen TG, Smith GS, Walford RL. Decline in mixed lymphocyte reactivity of spleen cells from aged mice of a long-lived strain. J Immunol 1973; 110: 1216–1221.
7. Chang MP, Makinodan T, Peterson WJ, Strehler BL. Role of T cells and adherent cells in age-related decline in interleukine-2 production. J Immunol 1983; 129: 2426–2430.
8. Gilchrest BA, ed. Skin and aging process. CRC press; 1984. p 67–82.
9. Sprecher E, Becker Y, Kraal G, Hall E, Harrison D, Shultz LD. Effect of aging on epidermal dendritic cell populations in C57BL/6J mice. J Invest Dermatol 1990; 94: 247–253.
10. Gilhar A, Aisen E, Pillar T, Eidelman S. Aged versus young skin response to intradermal administration of gamma interferon. J Am Acad Dermatol 1992; 27: 710–716.
11. Gilhar A, Aisen E, Pillar T, Etzioni A. Response to intradermal administration of gamma interferon of aged skin grafted onto nude mice. Dermatology 1996; 191: 99–103.
12. Sauder DN, Stanulis-Praeger BM, Gilchrest BA. Autocrine growth stimulation of human keratinocytes by epidermal cell-derived thymocyte activating factor: implications for skin aging. Arch Dermatol Res 1988; 2880: 71–76.
13. Gilhar A, Pillar T, David M. Aged versus young skin before and after transplantation onto nude mice. Br J Dermatol 1991; 124: 168–171.
14. Stanulis-Praeger BM, Gilchrest BA. Growth factor responsiveness declines during adulthood for human skin-derived cells. Mech Ageing Dev 1986; 35: 185–189.
15. Gilhar A, Pillar T, Etzioni A. Possible role of cytokines in cellular proliferation of the skin transplanted onto nude mice. Arch Dermatol 1995; 131: 38–42.
16. Gilhar A, Etzioni A, Moscona R. Topical cyclosporine induces hair growth in human split skin grafted onto nude mice. Acta Derm Venereol (Stockh) 1991; 71: 327–330.
17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156–159.
18. Weisinger G, DeCristofaro GD, LaGamma EF. Tissue- and treatment-specific regulation of multiple preproenkephalin transcriptional start site usage. J Biol Chem 1992; 267: 4508–4512.
19. Piechaczyk M, Blanchard GM, Marty L, Dani C, Panabieres FE, Sabouty S EL, et al. Post-transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression in rat tissues. Nuc Acids Res 1984; 12: 6951–6953.
20. Katz Y, Strunk RC. IL-1 and tumor necrosis factor: similarities and differences in stimulation of expression of alternative pathway of complement and IFN-1/IL-6 genes in human fibroblasts. J Immunol 1989; 142: 3862–3867.
21. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D. Tumor necrosis factor/cachetin interacts with endothelial cell receptors to induce release of interleukin-1. J Exp Med 1986; 163: 1363–1375.
22. Schroder J-M, Sticherling M, Henneicke HH, Preissner WC, Christophers E. IL-1 or tumor necrosis factor-alpha stimulate release of three NAP-1/IL-8 related neutrophil chemotactic proteins in human dermal fibroblasts. J Immunol 1990; 144: 2223–2232.
23. Kupper RS. Interleukin 1 and other immunologically active keratinocyte cytokines: molecular and functional characterization. Adv Dermatol 1988; 3: 293–305.
24. Deppert W, Buschhausen-Denker G, Patschinsky T, Steinmeyer K. Cell control of p53 in normal (3T3) and chemically transformed (Meth A) mouse cells. II. Requirement for cell cycle progression. Oncogene 1990; 5: 1701–1706.
25. Mercer WE, Shields MT, Lin D, Apella E, Ullrich SJ. Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. Proc Natl Acad Sci USA 1991; 88: 1958–1962.
26. Milner J. The role of p53 in the normal control of cell proliferation. Curr Opin Cell Biol 1991; 3: 282–286.
27. Bell E, Marek LF, Livingston DS. Loss of division potential in vitro: aging or differentiation? Science 1978; 202: 1158–1163.
28. Gilchrest BA. Age associated changes in the skin. J Am Geriat Soc 1982; 30: 139–143.
29. Grove GL, Kligman AM. Age-associated changes in human epidermal cell renewal. J Gerontol 1983; 39: 137–142.
30. Leyden JJ, McGinely KJ, Grove GL, Kligman AM. Age-related differences in the rate of desquamation of skin surface cells [Proceedings]. Adv Exper Med Biol 1978; 97.
31. Abb J, Abb H, Deinhardt F. Age-related decline of human interferon alpha and interferon gamma production. Blut 1984; 48: 285–289.
32. Belsito DV, Epstein SP, Schultz JM, Baer RL, Thorbecke GJ. Enhancement by various cytokines or 2-beta-mercaptoethanol or Ia antigen expression on Langerhans cells in skin from normal aged and young mice. J Immunol 1989; 143: 1530–1536.
33. Sauder DN. Effect of age on epidermal immune function. Dermatol Clin 1986; 4:
34. Thoman ML, Weigle WO. Lymphokines and aging: interleukin-2 production and activity in aged animals. J Immunol 1981; 127: 2102–2106.
35. Compton C, Tong Y, Trookman N, Zhao H, Roy D. TGF-beta-1 gene expression in cultered human keratinocytes does not decrese with biologic age. J Invest Dermatol 1994; 103: 127–133.
36. Gilhar A, Gershoni-Baruch R, Margolin A, Benderly A, Brandes JM. Dopa reaction of fetal melanocytes before and after skin transplantation onto nude mice. Br J Dermatol 1995; 133: 884–889.
37. Gilhar A, Ish-Shalom S, Pillar T, Etzioni A, Silbermann M. Effect of anti IGF-1 epidermal proliferation of human skin transplanted onto nude mice treated with growth hormone. Endocrinology 1994; 134: 229–232.