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In Vitro Paracrine Regulation of Human Keratinocyte Growth by Fibroblast-Derived Insulin-Like Growth Factors

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Human keratinocytes isolated from a skin biopsy and cultured in vitro on a feeder-layer of irradiated fibroblasts reconstitute a stratified squamous epithelium suitable for grafting onto patients suffering from large burn wounds. Since conditioned medium from 3T3-J2 cells can partially substitute for the intact feeder-layer, we studied the possible involvement of insulin-like growth factors acting in a paracrine fashion. IGFs were measured (after Sephadex G-50 gel-chromatography in acid conditions) in media conditioned by a feeder-layer of lethally irradiated 3T3-J2 fibroblasts on which keratinocytes were grown. Immunoreactive (IR) IGF-I, IGF-II, and IGF binding activity were present in the medium conditioned by the feeder-layer. The medium conditioned by keratinocytes showed nearly undetectable amounts of IR IGF-I and IGF-II, suggesting that keratinocytes are unable to synthesize IGFs peptides. Recombinant IGF-I and IGF-II, and conditioned medium from 3T3-J2 cells, caused a dose-dependent increase of ^{3}H-thymydine incorporation in cultured keratinocytes. The stimulatory effect of IGF and of 3T3-J2 conditioned medium was inhibited by the MoAb Sm 1.2, which recognizes both IGF-I and IGF-II but not insulin, and by the MoAb αIR-3, which is a specific antagonist of type-I IGF receptor. Fetal mouse-derived 3T3-J2 cells and adult human skin fibroblasts were equally able to sustain keratinocyte growth and in both cases addition of Sm 1.2 MoAb causes a 50% decrease in the keratinocyte number. When the non-IGF-producing BALB/c 3T3 cells were used as a feeder-layer, the keratinocytes number was similar to that observed with 3T3-J2 and with human fibroblasts plus the Sm 1.2 MoAb. IGF-I and IGF-II restored the BALB/c 3T3 growth promoting activity to the level of 3T3-J2 and of normal human fibroblasts. Our results suggest that fetal mouse 3T3-J2 and human fibroblasts synthesize IGF peptides, while keratinocytes do not. Fibroblast-derived IGFs stimulate keratinocyte growth in a paracrine fashion, suggesting their role in the regulation of keratinocyte proliferation in skin growth and in wound healing.

When human skin keratinocytes are cultivated on a feeder-layer of lethally irradiated fibroblasts (Rheinwald and Green, 1975), they form colonies that grow at the periphery, start to differentiate in their center, and eventually fuse, giving rise to a stratified squamous epithelium (Green et al., 1979) suitable for grafting onto patients presenting with large skin defects (Gallico et al., 1984; De Luca et al., 1989). Keratinocyte clonal growth, lifespan in culture, and maintenance of the proper differentiation are optimized when cells are grown on lethally irradiated fibroblasts of the 3T3-J2 cell line (Green, 1980), although other murine fibroblast cell lines or normal diploid human fibroblasts can support keratinocyte growth as well. As colonies grow, they gradually remove the feeder-layer from the surface of the vessel, so that fibroblasts are virtually absent in confluent cultures. Conditioned medium harvested from growing 3T3-J2 cells stimulates growth of normal human keratinocytes, but not of immortalized epithelial cells (Green, 1980). This suggests the possibility that these fibroblasts produce soluble growth factors that stimulate keratinocyte growth by a paracrine mechanism. The present study was undertaken to test the hypothesis that fibroblasts synthesize and release IGFs which then have the capacity to stimulate keratinocyte growth in a paracrine fashion.

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MATERIALS AND METHODS

Cell culture

Human keratinocytes were obtained from skin biopsies of healthy volunteers and cultivated on a feeder-layer of lethally irradiated 3T3-J2 cells, according to the method originally described by Rheinwald and Green (1975) (Green et al., 1979). In brief, 2 cm^2 skin biopsies were minced and trypsinized (0.05% trypsin and 0.01% EDTA) at 37°C for 3 hours. Cells were collected every 30 min, plated (1.8×10^6/75 cm^2 flask) on lethally irradiated 3T3-J2 cells, and cultured in 5% CO_2 in keratinocyte growth medium: Dulbecco-Vogt Eagle's and Ham's F12 media (3:1 mixture) containing 10% fetal calf serum (FCS), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (20 pM), epidermal growth factor (EGF, 10 ng/ml), glutamine (4 mM) and penicillin/streptomycin (50 IU/ml). In some experiments FCS, insulin, hydrocortisone, and EGF were omitted (basal medium). Subconfluent primary cultures were trypsinized and subcultured in secondary cultures as previously described (De Luca et al., 1988a). BALB/c 3T3 cells (clone A 31) were cultured in Dulbecco-Vogt Eagle's medium containing calf serum (10%), glutamine (4mM), and penicillin/streptomycin (50 IU/ml). Human fibroblasts were obtained from skin biopsies of healthy volunteers and cultured in Dulbecco-Vogt Eagle's medium containing calf serum (10%), glutamine (4mM), and penicillin/streptomycin (50 IU/ml). In some experiments subconfluent primary keratinocyte cultures were trypsinized and cells were cultured on lethally irradiated BALB/c 3T3 fibroblasts or on lethally irradiated normal human skin fibroblasts, as indicated in Results.

Analysis of the IGFs produced and released into the medium

Subconfluent cultures were washed three times in phosphate buffered saline (PBS) and placed in basal (serum-free) medium. After 24 hours the medium was replaced by fresh basal medium, and 48 hours later the conditioned medium was collected and processed.

The media conditioned by human skin fibroblasts, BALB/c 3T3 fibroblasts, non-irradiated 3T3-J2, lethally irradiated 3T3-J2, and by keratinocytes grown on the feeder-layer were acidified with 0.5 M acetic acid-0.15 M NaCl (pH of the mixture was < 3), and gel-filtered on Sephadex G-50 column (1.6×90 cm) equilibrated with 0.1 M acetic acid-0.15 M NaCl, pH 2.75. Fractions corresponding to 0–0.20, 0.21–0.40, 0.41–0.70, and 0.71–1 Kav were pooled, lyophilized, reconstituted in PBS, and analysed for IGF immunoreactivity and IGF specific binding activity.

IGF radioimmunoassay and IGF binding protein assay

IGFs were measured by radioimmunoassay using an antibody and ^{125}I-IGF-I provided by Nichols Institute (San Juan Capistrano, CA) for IGF-I, and a monoclonal antibody provided by Sera-lab (Techno-genetics, Trezzano S/N, Italy) and ^{125}I-IGF-II (Amersham, UK) for IGF-II; the standard curves were performed by recombinant Met-IGF-I and authentic IGF-II. The sensitivity of the assay is 90 pg/ml and the between assay coefficient of variation is 7.5%.

Specific binding of IGF-I was measured by overnight incubation at 4°C of 0.1 ml samples with about 20,000 CPM of (^{125}I)Thr^{59}-IGF-I (Amersham, UK), in 0.05 M Na phosphate buffer, pH 7.4, containing 2.5 g/l BSA in a final volume of 0.5 ml. Protein-bound IGF-I was separated by addition of 0.5 ml of a 20 g/l charcoal suspension in assay buffer containing 20 g/l BSA, and centrifugation at 3,000g for 20 min in a refrigerated centrifuge. Specific binding was determined in each sample as the difference between the radioactivity bound both in the absence and the presence of a saturating amount of partially purified IGF-I.

RNA blotting

Total RNA was prepared by the guanidine thiocyanate/cesium chloride method and enriched in poly A^+ RNA by oligo dT cellulose affinity chromatography (Lund et al., 1986). Poly A^+ RNA was size-fractionated on a 1% formaldehyde agarose gel. After electrophoresis, samples were transferred to Gene Screen membranes (New England Nuclear) and processed for Northern blot analysis (Lund et al., 1986). IGF-I and IGF-II cDNA probes (Jansen et al., 1983, 1985) were labelled with ^{32}P-dCTP (Amersham, 3,000 Ci/mmol) by random priming, and hybridization was performed for 48 hours at 42°C in a solution containing 50% formamide.

After hybridization, blots were washed six times in 2X SSC, 1% SDS, three times in 0.1X SSC at 65°C, and exposed to X-ray film (Kodak Xar-5) at −80°C with intensifying screens.

Growth assays

Keratinocytes from sub-confluent primary cultures were trypsinized and plated into 96-well microtiter plates in 0.2 ml of basal medium supplemented with 10% FCS. After 24 hours cells were washed three times and incubated for 18 hours in basal medium containing different reagents, as indicated in Results. ^{3}H-thymidine (5 Ci/mmol, Amersham, UK, 1.5 μCi/well) was added and cells were incubated for additional 18 hours. The radioactivity in the acid insoluble material was measured according to Russel et al. (1984). Every experiment was performed at least twice in triplicate.

To evaluate keratinocyte growth, cells (1×10^4/cm^2) were inoculated onto lethally irradiated fibroblasts (3T3-J2, BALB/c 3T3, or human skin fibroblasts; 4×10^4/cm^2) in insulin-free keratinocyte growth medium containing 0.5% FCS and different reagents, as indicated in Results. Six days later cells were trypsinized and counted in a hemocytometer. Every experiment was performed in triplicate.

RESULTS

Distribution of immunoreactive (IR) IGFs and IGF binding activity in conditioned media

Chromatographic analysis performed after acid-gel chromatography of conditioned media of different fibroblasts cell lines reveals the presence of two peaks of IGF-I and -II-like immunoreactivity. Figure 1 shows, for example, the IGF-I immunoreactivity and IGF-I binding capacity found in the medium of irradiated

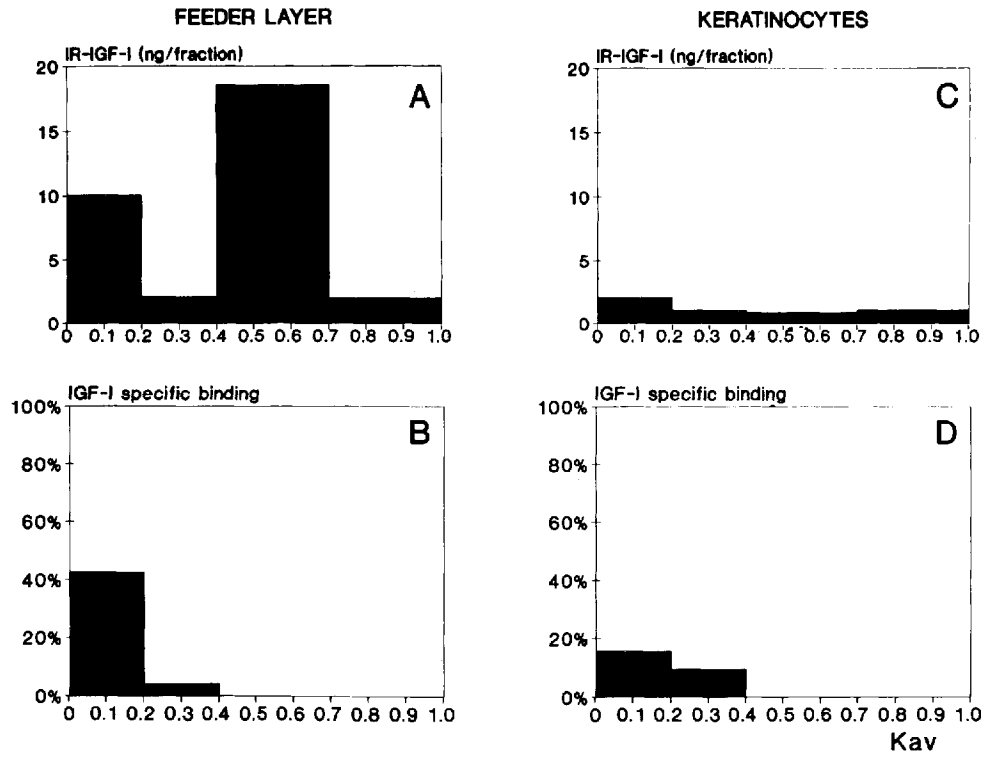

Fig. 1. Sephadex G-50 elution profiles of IR IGF-I (upper panels) and IGF-I binding capacity (lower panels) in conditioned media of 3T3-J2 fibroblasts **(panels A and B)** and of keratinocytes grown on 3T3-J2 **(panels C and D).** The conditioned media were acidified and gel-filtrated on Sephadex G-50 column equilibrated with 0.1 M acetic acid-0.15 M NaCl, pH 2.75. Fractions corresponding to 0–0.20, 0.21–0.40, 0.41–0.70, and 0.71–1 Kav were pooled, lyophilized, and reconstituted in phosphate buffered saline for IR IGF-I and IGF-I specific binding capacity analysis.

TABLE 1. IGF-I and IGF-II immunoactivities found after gel-filtration in acid conditions in media conditioned by non-irradiated and irradiated (feeder layer) 3T3-J2 fibroblasts, human skin fibroblasts, BALBc/3T3 fibroblasts and human keratinocytes[1]

RIA	3T3-J2	3T3-J2 irradiated	Human skin fibroblasts	BALB/c 3T3	Keratinocytes
IGF-I	8.90	5.00	31.20	0.05	0.02
IGF-II	8.10	5.51	30.60	0.64	0.14

[1]IGF values are expressed as ng per milliliter per 10^6 cells.

3T3-J2 cells. The first peak is located close to the void volume (Kav 0–0.20), corresponding to a higher molecular weight than that of IGF peptides. The second peak corresponds to the elution volume (Kav 0.41–0.70) of IGF-I (Fig. 1A). The first peak shows specific IGF binding activity (Fig. 1B), suggesting that the immunoreactivity found in the high molecular weight region is due, at least in part, to the interference in the RIA by the free binding sites.

In contrast to the IGF-I-like immunoreactivity and IGF binding activity present in the medium conditioned by irradiated 3T3-J2 fibroblasts, medium conditioned by human keratinocytes shows nearly undetectable levels of IGF-I-like immunoreactivity (Fig. 1C) and only a small amount of IGF binding activity (Fig. 1D).

In Table 1 are shown the IGF-I and IGF-II immunoactivities found in each medium tested in the second peak after acidic gel-filtration.

In agreement with these findings, Northern blot analysis evidenced the absence of expression of IGF-I and IGF-II in subconfluent and confluent keratinocytes and the expression of IGF-II mRNA in 3T3-J2 fibroblasts (Fig. 2).

Mitogenic effect of IGF-I and IGF-II on isolated keratinocytes

Exposure of keratinocytes to recombinant IGF-I and IGF-II produces a dose-dependent increase of ^{3}H-thymidine incorporation in the dose range of 5–25 ng of IGF/ml medium (Fig. 3). The stimulatory effect of IGF-I is abolished by the monoclonal antibody Sm 1.2 (Fig. 4), which recognizes both IGF-I and IGF-II, but not insulin (Maciel et al., 1988). The monoclonal antibody αIR-3, which is a specific antagonist of type-I IGF receptor, also inhibits IGF-I-induced ^{3}H-thymidine incorporation (Fig. 4). Both MoAbs exhibit their maximal activ-

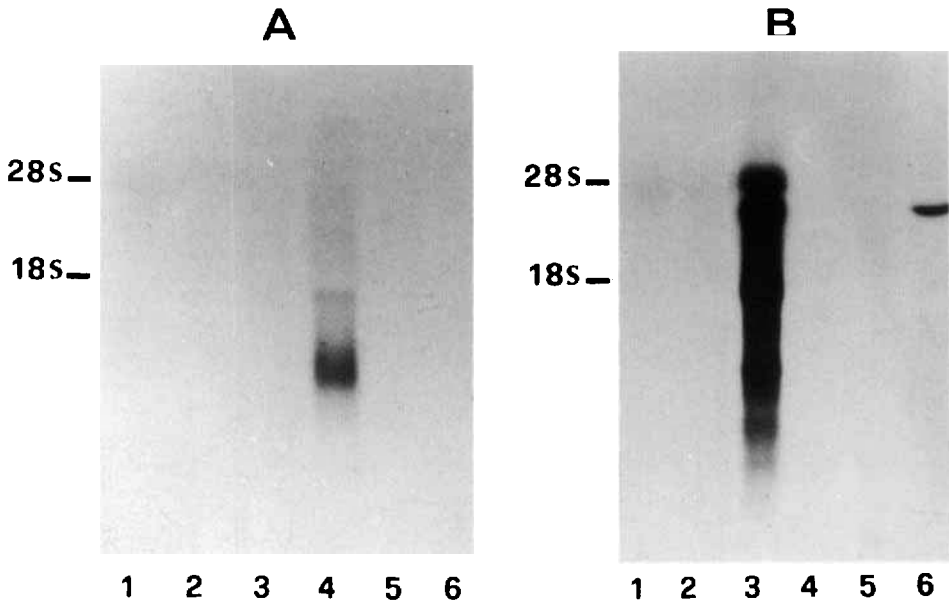

Fig. 2. Northern blot analysis of mRNA extracted from subconfluent (**lane 1**) and confluent (**lane 2**) keratinocytes, from fetal (**lane 3**) and adult (**lane 4**) rat liver, from BALBc/3T3 (**lane 5**) and 3T3-J2 (**lane 6**) fibroblasts. Gel **A** shows the autoradiogram obtained after hybridization with IGF-I cDNA probe; gel **B** shows the hybridization with IGF-II probe.

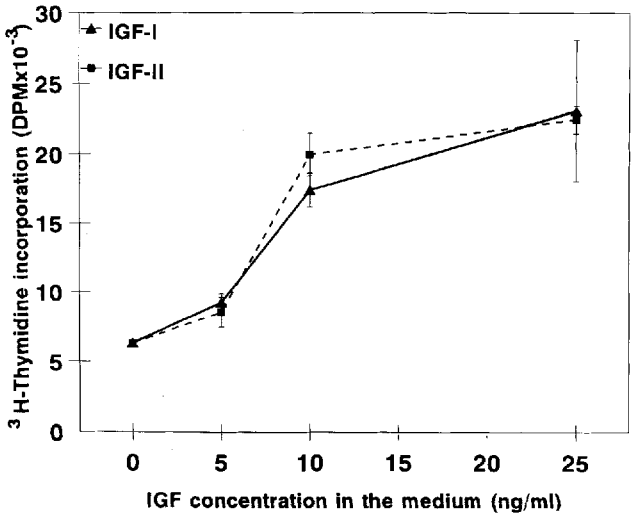

Fig. 3. Mitogenic effect of IGF-I and IGF-II on isolated keratinocytes. Keratinocytes from subconfluent primary cultures were trypsinized and plated at high density in basal medium supplemented with 10% FCS for 24 hours. Cells were then washed three times and the medium was replaced with basal medium containing increasing amounts of either synthetic IGF-I or IGF-II (5–25 ng/ml of basal medium).

ity at a concentration of 100 nmoles. A control unrelated MoAb (MAS) showed no effect (Table 2).

Additional evidence for a role of the type-I IGF receptor comes from the observation that ^{3}H-thymidine incorporation is increased by microgram amounts of insulin, the effect of 10 μg/ml of insulin being similar to that of 10 ng/ml of IGF-I. This stimulatory effect of insulin is inhibited by αIR-3, but not by Sm 1.2 MoAb (Table 2).

Mitogenic effect of 3T3-J2 and human skin fibroblasts on human keratinocytes

To confirm the role of IGFs in proliferation of keratinocytes, we incubated keratinocytes with increasing amounts of medium conditioned by lethally irradiated 3T3-J2 fibroblasts and observed a dose-dependent increase of ^{3}H-thymidine incorporation. This effect is evident at a 1% concentration of conditioned medium and reaches the maximum at 25% concentration (Fig. 5). The stimulatory effect of 25% concentration of conditioned medium is progressively inhibited by increasing amounts of the two MoAbs, Sm 1.2 and αIR-3 (Fig. 5).

Comparison of the growth promoting activity of the feeder-layers obtained from different fibroblast lines

The results obtained by the thymidine incorporation studies have been confirmed by estimating keratinocyte growth on feeder-layers of lethally irradiated 3T3-J2 cells, human diploid skin-derived fibroblasts, or BALB/c 3T3 cells. The activity of 3T3-J2 cells in sustaining keratinocyte growth is equivalent to that of human fibroblasts, and in both cases the addition of Sm 1.2 MoAb produces about 50% decrease of keratinocyte number (Fig. 6). When BALB/c 3T3 cells, which do not produce IGFs (Fig. 2, Table 1), are used as feeder-layer, the number of keratinocytes is similar to that observed with the 3T3-J2 and the human fibroblasts plus Sm 1.2 MoAb. The addition of 50 ng/ml of IGF-I or II restores the BALB/c 3T3 growth promoting activity to the level of the 3T3-J2 and the normal human fibroblasts (Fig. 6).

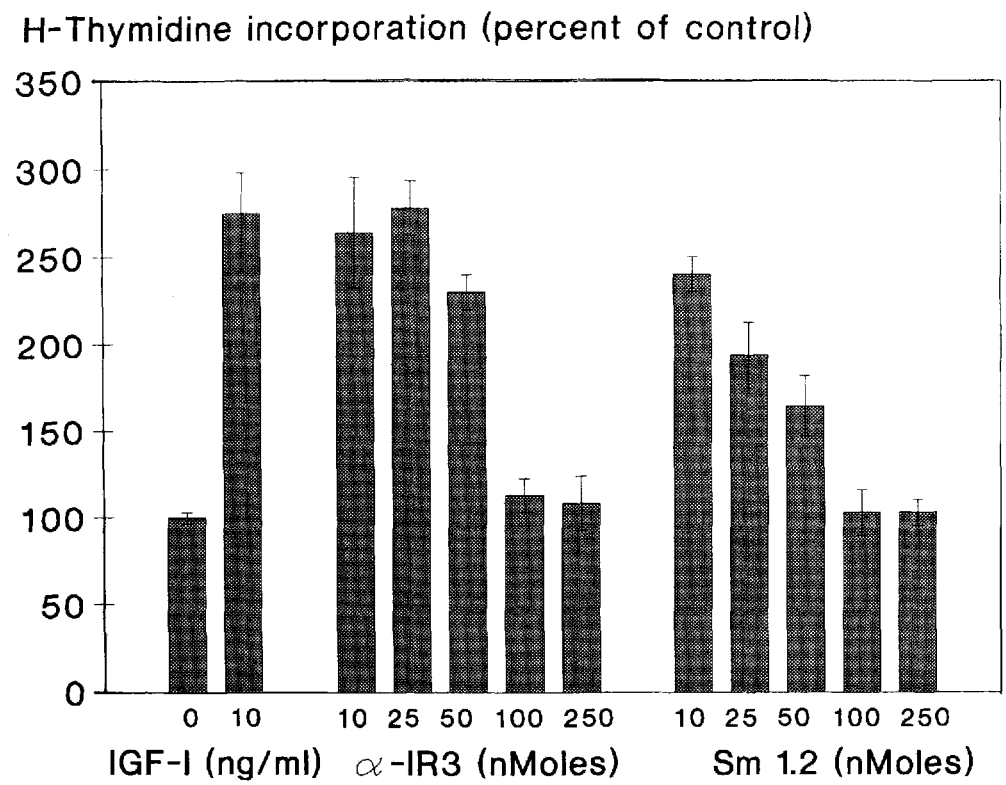

Fig. 4. Evaluation of the role of IGF-I on keratinocytes. Keratinocytes from subconfluent primary cultures were trypsinized and plated at high density in basal medium supplemented with 10% FCS for 24 hours. Cells were then washed three times and the medium was replaced with basal medium containing 10 ng/ml of IGF-I and increasing concentration of either αIR-3 or Sm 1.2 MoAbs. In the experiments where the αIR-3 MoAb was used, IGF-I was added 5 hours after the antibody. Results are expressed as the percent of basal medium (mean ± SEM).

TABLE 2. Effect of anti-IGF antibodies on IGF-I and insulin-stimulated thymidine incorporation by isolated keratinocytes[1]

	Control	αIR-3 (100 nMol)	Sm 1.2 (100 nMol)	MAS (100 nMol)
Basal medium				
n	12	6	6	6
mean	100	98.6	102.54	118.34
SE	3.33	4.53	11.44	14.63
P vs. control		n.s.	n.s.	n.s.
Insulin 10 mg/ml				
n	12	11	12	
mean	225.54	121.54	231.39	
SE	40.05	5.22	105.34	
P vs. control		0.025	n.s.	
IGF-I 10 ng/ml				
n	18	6	6	9
mean	275.81	113.13	103.19	262.29
SE	19.54	9.63	13.24	11.76
P vs. control		< 0.0001	< 0.0001	n.s.

[1]Keratinocytes from subconfluent primary cultures were trypsinized and plated at high density in basal medium supplemented with 10% FCS for 24 hours. Cells were then treated as described in the legend to Figure 4 and in Materials and Methods. The results represent the pooled data from at least two experiments in each case. Significance values were obtained using Students' unpaired t-test.
n.s. = not significant.

DISCUSSION

Interactions between epithelial cells and mesenchyme are important for growth and regulation of differentiation of normal epithelia (Ekblom et al., 1986). A suitable in vitro model for the study of such interactions is represented by the skin keratinocytes grown on a feeder-layer of irradiated fibroblasts (Green et al., 1979). Under these conditions, keratinocyte colonies give rise to a tri-dimensional tissue which maintains keratinocyte differentiation (Green, 1980; Asselineau et al., 1989), interactions among cells present in the epidermis (De Luca et al., 1988a, 1988b) and interactions between basal keratinocytes and basement membrane (De Luca et al., 1990; Marchisio et al., 1991). Here we show that mouse fetal 3T3-J2 cells and human adult skin fibroblasts stimulate keratinocyte growth by the paracrine synthesis and release of IGF peptides.

The finding of an IGF-II expression in fetal 3T3-J2 fibroblasts is in agreement with previous reports showing a developmentally regulated expression of IGF-II and IGF-I in rodents (Adams et al., 1983; Lund et al., 1986). The expression of IGF-II predominates in fetal life and shifts to expression of IGF-I in postnatal life. The finding of IGF-I-like immunoreactivity in media conditioned by 3T3-J2 fibroblasts contrasts with the lack of detection of its mRNA. An extremely high turnover of the IGF-I mRNA is envisaged. However, we do not have at the moment any definitive explanation.

Pure recombinant IGF-I and II and the fibroblast-conditioned medium are mitogenic on isolated keratinocytes, and the mitogenic effect is inhibited by αIR-3 MoAb, suggesting that in normal epithelial cells both IGF-I and IGF-II exert their growth promoting activity

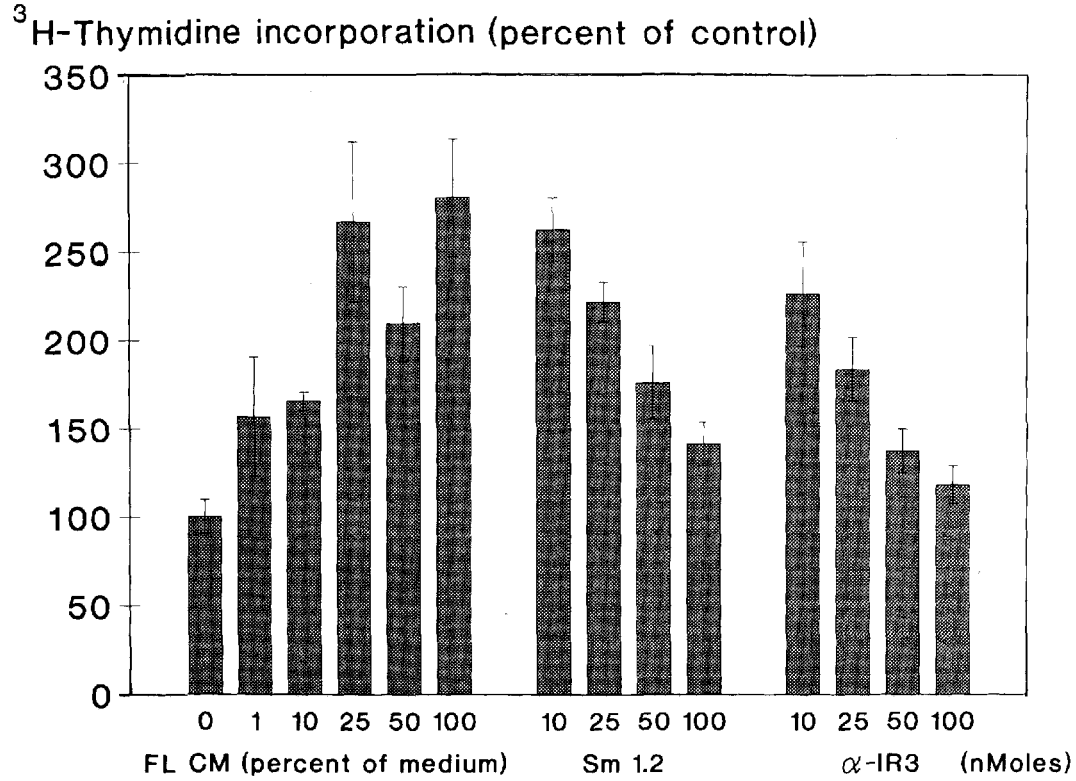

Fig. 5. Mitogenic effect of 3T3-J2 conditioned media on isolated keratinocytes. Keratinocytes from subconfluent primary cultures were trypsinized and plated at high density in basal medium supplemented with 10% FCS for 24 hours. Cells were then washed three times and the medium was replaced with basal medium containing increasing amounts of medium conditioned by the feeder-layer of lethally irradiated 3T3-J2 fibroblasts. In subsequent experiments the medium was replaced with basal medium containing 25% conditioned medium and an increasing concentration of either αIR-3 or Sm 1.2 MoAbs. Results are expressed as percent of basal medium (mean ± SEM).

through type-I IGF receptors, in agreement with the finding of Neely et al. (1991). The demonstration that a MoAb of the same class of the αIR-3 does not affect the IGF-I-stimulated thymidine incorporation diminishes the likelihood of a non-specific toxic effect of the αIR-3. Similarly, the inhibitory effect of the anti-IGF Sm 1.2 antibody is overcome by high concentrations of insulin, ruling out a non-specific toxicity of this antibody (Table 2). The progressive inhibition of thymidine incorporation by increasing amounts of anti-IGF or anti-type-I IGF receptor antibodies shows that IGFs are responsible to a considerable extent for the mitogenic activity contained in the medium. However, the keratinocyte growth stimulated by the feeder-layer conditioned medium is not completely inhibited by the two MoAbs studied. Moreover, non-IGF producing BALB/c 3T3 cells are able to partially sustain keratinocyte growth. This suggests that the paracrine effect of IGFs is only one of the mechanisms involved in keratinocyte stimulation by fibroblasts. In fact, it has been shown that keratinocytes synthesize and secrete several cytokines. Some of these factors, such as transforming growth factor (TGF) α and β and bFGF also may have a role in the autocrine regulation of keratinocyte migration and proliferation (Barrandon and Green, 1987; Coffey et al., 1987; Kane et al., 1989; Halaban et al., 1988).

Our data indicate that normal human epithelial cells do not synthesize IGFs. The observations that breast

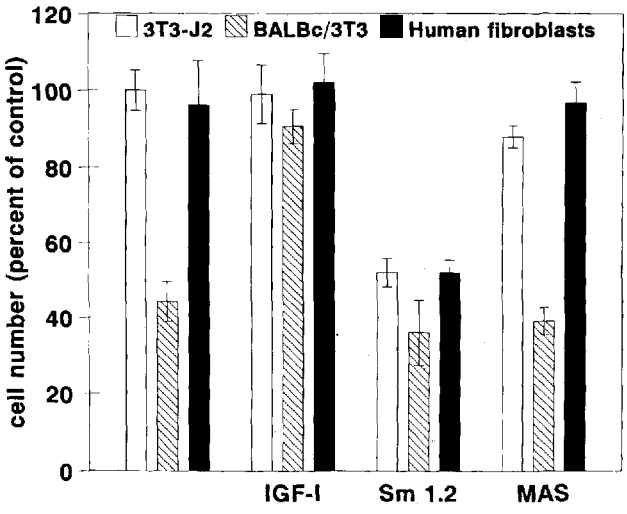

Fig. 6. Comparison of the growth potency of the feeder-layers obtained by 3T3-J2, BALB/c 3T3, and human skin fibroblasts. Keratinocytes (1×10^4/cm^2) were inoculated in duplicate into the plates containing irradiated fibroblasts (3T3-J2, BALB/c 3T3, human skin; 4×10^4/cm^2), in insulin-free keratinocyte growth medium containing 0.5% FCS in the absence or presence of 50 ng/ml of IGF-I with or without 200 nM of Sm 1.2 or MAS MoAbs. Six days later cells were trypsinized and counted in a hemocytometer.

and lung tumor cells synthesize large amounts of IGF-I and IGF binding proteins in vivo (Minuto et al., 1986) and in vitro (Minuto et al., 1987, 1988) supports the hypothesis of an IGF-dependent autocrine stimulation of cancer cells of epithelial origin.

As shown by Stiles et al. (1979) on quiescent fibroblasts, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) induce "competence" of cells to replicate their DNA and divide. Competent cells, however, do not "progress" efficiently into the S phase of the cell cycle unless they are incubated in the presence of IGFs or EGF. The same mechanism is involved in the IGF stimulation of keratinocyte growth, since keratinocytes require exposure to PDGF or a minimal amount of serum in order to respond to IGFs, both in terms of thymidine incorporation and cell number (not shown). It has been shown by Clemmons (1984) that PDGF is an important regulator of IGF production by human skin fibroblasts. It is tempting, therefore, to speculate that, in the cascade of paracrine events involved in wound healing, the PDGF released by platelets during blood clotting stimulates IGF-I synthesis by fibroblasts and makes keratinocytes "competent" to IGF-I growth promoting activity.

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