Specific effects of ras oncogene expression on the growth and histogenesis of human epidermal keratinocytes

Denis R. Henrard¹, Alan T. Thornley², M. Liesbeth Brown & James G. Rheinwald

Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, and Department of Cellular and Molecular Physiology, Harvard Medical School, 44 Binney Street, Boston Massachusetts 02115, USA

Little progress has been made in identifying specific regulatory pathways that might be affected in cells by a mutationally activated p21ras when its expression does not lead to complete transformation. We wished to determine whether a normal, diploid human epithelial cell in which activation of ras had occurred could be identified in culture and, furthermore, whether expression of a mutant p21ras in such an otherwise normal cell would result in abnormal histogenic behavior in vivo. Thus, we introduced the v-Ha-ras gene into an early passage culture of normal human epidermal keratinocytes via a defective retrovirus. We examined these genetically engineered cells for changes in growth and differentiation, both in culture and in the epithelium formed when cultures were grafted to the skin of nude mice. We have found that keratinocytes expressing p21ras are independent of epidermal growth factor (EGF) — a factor which is normally essential for the growth of keratinocytes but that they are otherwise indistinguishable in culture from normal cells. v-ras keratinocytes also secrete a factor possessing some specific biological activities of members of the fibroblast growth factor (FGF) family, which is distinct from acidic and basic FGF. In short-term culture grafts the v-ras cells form a non-invasive and normally differentiating epithelium. However, the cells express elevated levels of keratin 19, which is a characteristic of fetal epidermis and of premalignant lesions of some stratified squamous epithelia.

Introduction

Oncogenes of the ras family have a dominant transforming effect when experimentally introduced into many established cell lines (for review, see Barbacid, 1987). In diploid cells, however, expression of a ras oncogene typically does not cause malignant transformation (Sager et al., 1983; Tubo & Rheinwald, 1987); the most consistent phenotypic change observed in culture is a reduction of mitogen dependency (Tubo & Rheinwald, 1987; Zhan et al., 1987). A mutant Ha-ras has been found to induce EGF-independence in several epithelial cell types which normally require exogenous EGF for optimal growth in culture (Weissman & Aaronson, 1983; Tubo & Rheinwald, 1987; Falco et al., 1988). In a few instances, cell populations which had been transfected with a ras oncogene have also exhibited an extended replicative lifespan in vitro (Tubo & Rheinwald, 1987) or have ultimately given rise to an immortal cell line (Spandidos & Wilkie, 1984; Yoakum et al., 1985). However, such immortal variants were invariably aneuploid; thus, it is likely that secondary genetic alterations had occurred in the subpopulation that escaped senescence.

The in vivo effects of expressing a ras oncogene in cells that do not become tumorigenic as a consequence is usually impossible to evaluate; such cells neither grow progressively nor form a recognizable, even in transient, tissue structure upon standard subcutaneous injection. However, the keratinocyte is a cell type that is quite amenable to histogenic evaluation. When grafted to the skin of nude mice, even normal cultured cells of this type organize into an epithelium which differentiates correctly (Worst et al., 1974; Barrandon et al., 1988). A wide range of clinically recognized benign and reneoplastic lesions of human stratified squamous epithelia have been characterized extensively (Carter, 1984). Thus, any histologic abnormality evident in grafts of human keratinocytes expressing an activated ras should be classifiable with respect to known premalignant phenotypes.

We report here that human epidermal keratinocytes expressing v-Ha-ras display EGF-independence and secrete an FGF-like factor in culture. In vivo, the cells differentiate normally but express elevated levels of keratin 19 — a property shared by fetal epidermis (Banks-Schlegel, 1982; Moll et al., 1983; Dale et al., 1985) and premalignant lesions of some stratified squamous epithelia (Lindberg & Rheinwald, 1989).

Results

Human keratinocytes expressing p21v-Ha-ras are EGF-independent for growth in culture

We used the defective amphototropic retrovirus Zipneo/ ras (Feig et al., 1977) to introduce the v-Ha-ras gene into the normal human epidermal keratinocyte strain YF29. Our previous study of normal human mesothelial cells (Tubo & Rheinwald, 1987) and a study by Weisssman and Aaronson (1983) of the mouse epithelial cell line Balb/MK disclosed that expression of an oncogenic Ha-ras induces EGF-independence in these epithelial cell types. Thus, we compared growth of the G418-resistant (G418*) colonies arising after Zipneo/ras infection in the presence of absence of EGF. As shown in Figure 1, the infection frequency, growth rate, and colony morphology of the G418* Zipneo/ras transfectants were essentially identical in the presence or absence of EGF. Colonies of v-ras-keratinocytes did not stratify in the absence of EGF, in contrast to normal keratinocytes which form thick multilayers of cornified cells at the expense of progressive multiplication when
deprived of EGF (Reinwald & Green, 1975; 1977; Barrandon & Green, 1987). The v-ras-keratinocytes could also be subcultured and serially passaged in the absence of EGF. In contrast, the parent YF29 cells infected with a control retrovirus (neuMLV) (Kaplan & Eckhart, 1987), which expresses only a neu gene, stratified extensively, grew very slowly, and could not be serially subcultured without EGF.

In order to assess the properties of typical v-ras cells, we pooled about 100 G418 colonies that arose after infection with Zipneo/ras (the 'YF-ras' pool) and compared these YF-ras cells with a pool of about 1000 G418 colonies obtained after infection with neuMLV (the 'YF-neo' pool). As shown in Figure 2, YF-ras cells expressed the viral, mutant form of p21\textsuperscript{ras} at levels equivalent to that of the endogenous protein.

Aside from their EGF-independence in culture, YF-ras cells were indistinguishable from normal keratinocytes in their dependence upon fibroblast feeder cells and other stimulatory factors such as cholera toxin and adenosine, in their sensitivity to growth inhibition by TGF-beta and the phorbol ester TPA, and in their sensitivity to terminal differentiation induced by transient anchorage-deprivation (see Reinwald & Beckett, 1980) (data not shown). Interestingly, YF-ras cells grew extremely poorly in an alternate, serum- and feeder-free keratinocyte culture medium (MCDB153 supplemented with pituitary extract and other growth factors (Peel & Ham, 1980; Boyce & Ham, 1983)), whereas YF-neo and the parent YF29 cells grew well in this medium. Both YF-ras and YF-neo pools grew for 40-50 cell generations after transduction and then senesced after achieving the replicative lifespan characteristic of YF29, without the emergence of an immortal cell line.

**V-ras-keratinocytes secrete an FGF-like factor**

Another epithelial cell type studied by this laboratory, the mesothelial cell, exhibits a stringent requirement for EGF or FGF for growth in culture (Connell & Reinwald, 1983; Zenzie & Reinwald, in preparation). In contrast, EJras- or v-Ha-ras-transfected mesothelial cells are EGF/FGF-independent and secrete a growth factor that can satisfy the mitogenic function of EGF or FGF for normal mesothelial cells (Tubo & Reinwald, 1987; Zenzie & Reinwald, in preparation). Recent experiments have disclosed that the activity secreted by ras-mesothelial cells is not EGF or TGF-alpha, but instead is a factor that has some biological activities of members of the FGF family (Zenzie & Reinwald, in preparation). Thus, we examined the YF-ras keratinocytes for secretion of a similar activity, using clonal growth stimulation of normal mesothelial cells as a sensitive and specific bioassay for the presence of such a mitogen in YF-ras conditioned medium (CM). (Mesothelial cell growth is greatly, and specifically, stimulated by EGF, TGF-alpha, basic FGF, acidic FGF, and K-FGF, whereas all other factors tested, including IL-1, G-CSF, GM-CSF, GM-CSF, IGF-1, TGF-beta, PDGF, and TNF, cannot replace EGF or FGF as mitogens for normal mesothelial cells (Connell & Reinwald, 1983; Chang, Zenzie & Reinwald, in preparation.) As shown in Figure 3a, YF-ras CM was consistently better in promoting growth of mesothelial cells than was YF-neo CM. Interestingly, this YF-ras mitogenic activity was inhibited by heparin, which also
inhibited the activity of basic FGF, but not that of EGF, TGF-alpha, or αFGF in this assay (Figure 3; Zenzie & Rheinwald, in preparation). Thus, the YF-ras factor exhibited a distinctive characteristic of a member of the Fibroblast Growth Factor family.

We next determined whether the YF-ras factor had another specific biological activity of FGFs. Cells of the rat pheochromocytoma line PC12 extend neurites in response to FGFs as well as to Nerve Growth Factor (NGF) (Wagner & D'Amore, 1986; Neufeld et al., 1987). As shown in Figure 3b, YF-ras CM but not YF-neo CM induced PC12 neurite outgrowth. The time of appearance of neurites after exposure to YF-ras CM was more similar to that of FGFs (4 days) than of NGF (2 days). In addition, heparin promoted a substantial lengthening of the processes in response to YF-ras CM (Figure 3b inset) but not to NGF. Thus, the activity contained in YF-ras CM is related to FGF and not to NGF. An antiserum (Sato & Rifkin, 1988) that completely neutralized 3 ng ml⁻¹ of basic FGF did not affect the PC12 response to YF-ras CM (Figure 3b), indicating that the YF-ras factor is not basic FGF. The way in which heparin modulated the activity of the YF-ras factor also indicated that it was not acidic FGF: heparin greatly potentiated the activity of αFGF in both the mesothelial and PC12 assays, whereas the YF-ras CM activity was inhibited by heparin for mesothelial cells and enhanced for PC12 cells (Figure 3). A number of new factors related to acidic or basic FGF have been reported recently (Delli-Bovi et al., 1988; Zhan et al., 1988; Rubin et al., 1989) and we are currently trying to characterize the YF-ras factor precisely.

Expression of keratin 19 is abnormal in the epithelium formed by YF-ras cells in vivo

Cell lines cultured from malignant human squamous cell carcinomas (SCC cells) share with YF-ras cells the in vitro properties of EGF-independence (Rheinwald & Beckett, 1981) and poor growth in MCDB153 medium (Rollins et al., 1989). However, we found that, unlike SCC cells, YF-ras cells were not tumorigenic in nude mice (no lump or tumor arose from subcutaneous injections of 1–3 × 10⁶ cells into four mice). In order to determine to what extent the histogenic potential and
epithelia, either is not expressed or is expressed at low levels, confined to the basal, proliferative cell layer (Banks-Schlegel, 1982; Dixon & Stanley, 1984; Dale et al., 1985; Morgan et al., 1987a; Bosch et al., 1988).

In control YF29 or YF-neo grafts, K19 expression was largely absent or occasionally present in patchy areas restricted to the basal layer (Figure 5a, a'). However, in the epithelium formed by grafts of YF-ras keratinocytes (Figure 5b, b'), K19 was detected in over 95% of the basal cells and in 50-60% of the suprabasal cells. In the epithelium formed by a single YF-ras clone (YF-ras clone A), originally picked because of its particularly good growth in the G418 selection plate, K19 was expressed almost uniformly in all cell layers of grafts (Figure 5c). (An epidermal squamous cell carcinoma-derived line, SCC-13, (Rheinwald & Beckett, 1981) formed within one week of grafting an epithelium that also exhibited a suprabasal, variegated pattern of K19 expression (Figure 5). However, in contrast to the behavior of YF-ras cells, the epithelium was severely dysplastic and hyperplastic. The pattern of K19 expression in YF-ras grafts was reminiscent of that of proneoplastic lesions of oral stratified squamous epithelium (Lindberg & Rheinwald, 1989): the cells differentiated normally, were not hyperplastic, and did not invade the graft bed, yet exhibited increased expression and altered localization of K19.

**Discussion**

There is growing evidence that c-Ha-ras mutation can play a role early in the process of epithelial neoplasia. This was first suggested by Quintanilla et al. (1986) who reported that, after treatment of mouse epidermis with dimethylbenzanthracene and repeated application of the tumor promoter TPA, a high proportion of the papillomas that developed consisted of cells bearing a codon 61 mutation in the c-Ha-ras gene. Direct evidence for a causal role of ras mutation in this system came from a later study by the same laboratory (Brown et al., 1986), which reported that Ha-MuSV infection of mouse skin served to initiate epidermal carcinogenesis. Recently, it has been found that introduction of a human Ha-ras oncogene into midgestation mouse embryos via defective retroviral infection results in extensive epidermal hyperplasia as well as growth of largely benign papillomas and cystic skin lesions in the adult animal (Compere et al., 1989). Finally, it has been reported that a high proportion of benign, spontaneously regressing human epidermal lesions (keratoacanthomas) contains an activated Ha-ras gene (Corominas et al., 1989). Our results have identified several specific changes in cell behavior that result from expression of an activated p21ras in otherwise normal human epidermal cells. Any or all of these changes could, by their nature, play an important role in initiating the multistep process of carcinogenesis in this tissue.

Our finding that expression of a ras oncogene causes normal human keratinocytes to become EGF-independent for growth in culture is consistent with our earlier study of the effect of Edras in normal human mesothelial cells (Tubo & Rheinwald, 1987) and...
with those of Aaronson and colleagues (Weissman & Aaronson, 1983; Falco et al., 1988) in the established mouse epidermal cell line Balb/MK. Our results with diploid human cells differ, however, from those of Aaronson and also from those of Yuspa et al. (1985): mouse epidermal cells infected with Ha-MuSV are Ca\(^{2+}\)- and TPA-resistant while our ras human cells are not altered in these characteristics. Additionally, our ras human epidermal cells secrete an FGF-like mitogen, whereas the secreted factor was typically detected in the mouse system (Falco et al., 1988). Expression of a ras oncogene has been reported to result in mitogen-independence and induction of possible FGF-related factors in other human cell types—namely, mesothelial cells (Tubo & Rheinwald, 1987; Zenzie & Rheinwald, in preparation), endothelial cells (Faller et al., 1988), and the mammary carcinoma cell line MCF-7 (M.E. Lippman, personal communication). Thus, either species differences or an inability to detect FGF-type molecules in the mouse system must be invoked to account for the differences observed between the two species.

Cells cultured from fully malignant squamous cell carcinomas (SCC cells) resemble the YF-ras keratinocytes in that they are EGF-independent and do not stratify in culture (Rheinwald & Beckett, 1981). SCC cells are also defective in the mechanism that triggers terminal differentiation in response to external stimuli, such as anchorage-deprivation (Rheinwald & Beckett, 1980) and TPA (reviewed by Parkinson, 1985). This had suggested a possible link between EGF-dependence and a normal terminal differentiation mechanism in keratinocytes. Earlier work had demonstrated that EGF does not act as a keratinocyte mitogen per se, but rather helps to maintain progressive, rapid multiplication of cells in large colonies by promoting centrifugal migration. As a result, colony stratification and consequent suprabasal cell differentiation are greatly reduced (Rheinwald & Green, 1977; Barrandon & Green, 1987). Our EGF-independent, YF-ras keratinocytes remain normally sensitive to TPA and to transient anchorage-deprivation, however, indicating that expression of v-ras does not directly affect the terminal differentiation ‘switch’ in normal human keratinocytes. YF-ras cells fail to stratify in culture apparently because they have become EGF-independent for cell migration and colony expansion. These results clearly indicate separable mechanisms for an EGF-modulated regulatory pathway and for the control of commitment to terminal differentiation in the keratinoocyte.

In vivo, human v-ras keratinocytes formed an essentially normal tissue and expressed two suprabasal markers of epidermal differentiation, involucrin and filaggrin, at levels similar to those found in control grafts. The thickness of the epithelium formed by YF-ras cells was similar to that formed by control cells, providing no evidence of hyperplastic behavior. In contrast, primary cultures of mouse epidermal cells infected with another defective retrovirus bearing the v-Ha-ras oncogene formed papillomas when grafted to mouse skin (Roop et al., 1986). We grafted human epidermal cells as intact sheets, while Roop et al. inoculated single cell suspensions of mouse epidermal cells into a chamber mounted on the skin. Thus the apparent discrepancy in results between the two systems might reflect either species differences or a difference between the two grafting systems with respect to the in vivo environment seen by the cells. In support of the latter possibility, Brown et al. (1986) have found that mouse epidermis directly infected with Ha-MuSV did not develop papillomas; the epidermis remained histologically normal while containing ‘initiated’ cells which could grow into papillomas in response to repeated applications of TPA.

A role for ras mutation as an event that could initiate carcinogenesis in human stratified squamous epithelia is further supported by the abnormal expression of keratin 19 by YF-ras cells in vivo. Keratin 19 is not present in normal adult epidermis, but is expressed in the basal cells of normal fetal epidermis (Banks-Schlegel, 1982; Dale et al., 1985). A low level of K19 expression is retained in basal cells of normal keratinized squamous epithelia in the adult (Dixon & Stanley, 1984; Bartek et al., 1986; Morgan et al., 1987a; Bosch et al., 1988; Lindberg & Rheinwald, 1989). This laboratory has reported recently that suprabasal K19 expression is a consistent property of preneoplastic lesions of the oral stratified squamous epithelia (Lindberg & Rheinwald, 1989). The almost uniform expression of K19 in the basal layer of grafts of YF-ras epidermal cells is consistent with a return to a fetal pattern of expression. However, the significant suprabasal expression of K19 in these grafts suggests a preneoplastic, ‘initiated’ state of these cells, in which a subsequent mutation(s) could result in formation of an overtly abnormal, dysplastic or invasive lesion.

A very recent paper by Ryle et al. (1989) has reported an increase in K19 expression in culture resulting from expression of a ras oncogene in the established human epidermal cell line HaCaT. However, the parental cell line is aneuploid and its growth regulation and pattern of keratin expression differs from that of normal diploid keratinocytes. Thus, the ras-HaCaT cells should be considered as being at a later stage of neoplastic progression than the YF-ras cells we have described here. We do not know how keratin 19 becomes expressed abnormally in premalignant lesions of some stratified squamous epithelia nor how it becomes induced in normal epidermal cells as a result of ras mutation. The gene encoding K19 has been cloned (Badner et al., 1986; Eckert, 1988), so it is now feasible to pursue the molecular mechanism of keratin 19 induction by mutant p21**. Understanding this mechanism and learning the identity of the secreted mitogen which is also induced in response to ras mutation should provide critical insights into the role of this oncogene in epithelial growth regulation and neoplasia.

Materials and methods

Epidermal cells, culture medium, and growth factors

The normal human epidermal keratinocyte strain YF29 (a gift from Y. Barrandon and H. Green, Harvard Medical School) were cultured from newborn foreskin, using methods and medium described previously (Rheinwald & Green, 1975; Allen-Hoffman & Rheinwald, 1984). Epidermal cells were
Cocultivated with lethally irradiated 3T3-J2 feeder cells in a medium consisting of a 1:1 mixture of the nutrient media DME and F12 (SIGMA) supplemented with 5% fetal calf serum, 0.4 μg ml⁻¹ hydrocortisone, 5 μg ml⁻¹ insulin, 10⁻⁶ M cholera toxin, 1.8 × 10⁻⁴ M adenosine, and 10 ng ml⁻¹ epidermal growth factor (EGF).

Recombinant human growth factors (EGF and TGF-alpha from Chiron Corp., basic FGF from Ethicon Corp., and acidic FGF from Dr. T. Maciag) were prepared as concentrated stock solutions in HEPES-buffered Earle's salts (HBES) + 0.1% bovine serum albumin (BSA) and were stored at -20°C. Heparin (porcine intestinal mucosal, Grade I, SIGMA) was prepared as a 3 mg ml⁻¹ (100 x) stock solution in HBES + BSA each week and was stored at 4°C.

Transduction using defective retroviruses

Amphotrophic producer cell lines were generated by retroviral infection of PA317 cells (Miller & Buttimore, 1986) with supernatants of ecotropic virus-producing psi2 cells (Mann et al., 1983) that had previously been transsected with the pneoMLV (Kaplan & Eckhart, 1987) or the pZIPneo/ras (Feig et al., 1987) plasmids. 1–2 × 10⁵ cells of the highest titer PA317 producer clones yielded 10⁸ (Zincos/neo) and 10⁷ (neom) infectious particles per ml of culture supernatant, as assayed on NIH3T3 cells (Miller & Buttimore, 1986). To use for transduction of epidermal cells, PA317 producer cells were lethally irradiated and cocultivated with 5 × 10⁸ YF29 cells in the presence of 4 mg ml⁻¹ polybrene (SIGMA), as described (Morgan et al., 1987b). After 2 to 3 days, the PA317 producer cells were selectively removed by brief incubation with an EDTA solution and were replaced by lethally irradiated 3T3J2 cells into which the neo gene had been previously introduced by transfection. Cultures were re-fed every 3 days with medium containing 0.25 mg mg⁻¹ active G418 (Geneticin, Gibco), either in the presence or absence of 10 ng ml⁻¹ EGF. After 10–14 days, photographs of representative colonies were taken, some dishes were fixed and stained with methylene blue, and some dishes were subcultured.

Electrophoresis and electrophoretic map

Whole cell extracts were prepared by lysing near-confluent cultures in Laemmli buffer, 25–50 micrograms of extract, as determined by the Biorad protein assay, were separated electrophoretically on a 12.5% polyacrylamide gel, blotted onto a nitrocellulose filter using a Polyblot apparatus (American Bioanalytics) and reacted with the mouse ras 10 antibody (Dupont, Inc.), which recognizes both normal and mutant forms of p21^H^H. Immunoreactive bands were detected by the avidin-biotin alkaline-phosphatase method (Vector Laboratories, Burlingame, CA).

Mitogenesis and differentiation assays

Subconfluent, 100 mm cultures of YF-neo or YF-ras cells were rinsed with PBS and cultured for an additional 16 h in 5 ml of DME/F12 supplemented with 1% serum. The conditioned medium (CM) was made 20 mM in HEPES and either was sterile-filtered or was first concentrated 10-fold using an Amicon YM10 ultrafiltration membrane. CM was stored at -20°C until use.

Normal human mesothelial cell strain LP-9 was used as a bioassay for EGF-like and FGF-like mitogens, as described (Tubo & Rheinwald, 1987; Zenie & Rheinwald, in preparation). Cells were plated at 10⁴ per 60 mm dish in 4.5 ml of mesothelial cell growth medium (Connell & Rheinwald, 1983; Tubo & Rheinwald, 1987), consisting of a 1:1 mixture of M199 and MCDB105 nutrient media (SIGMA) supplemented with 10% calf serum and 0.4 μg ml⁻¹ hydrocortisone, but lacking EGF. Either 0.5 ml of CM or a final concentration of 10 ng ml⁻¹ purified growth factor was then added ±30 μg ml⁻¹ heparin. Cultures were re-fed after 7 and 10 days and were fixed and stained on day 14.

The rat pheochromocytoma line PC12 (Green & Tischler, 1976) was used to assess neurite promoting activity. Cells were plated at 10³ per well in 24-well dishes with 1.5 ml of PC12 growth medium (DME supplemented with 10% calf serum and 5% heat-inactivated horse serum), and either 75 μl of concentrated CM or 1 ng ml⁻¹ of a purified growth factor was added. Cultures were re-fed after two days and scored after 8 days for the per cent of cells possessing processes extending at least twice their diameter.

Xenografting cultured epidermal cells and immunohistological evaluation

Confluent cultured cell sheets were grafted to the underside of the dermis of NIH Swiss or Balb/C nu/nu mice using a minor modification of the inverted flap method described by Barrandon et al. (1988). Cells were plated at 2 × 10⁵/60 mm dish and grown to confluence. (As described earlier (Rheinwald & Green, 1975), in this culture system the epithelial cells attach directly to the plastic culture vessel surface and the 3T3 feeder cells are displaced as the colonies expand laterally.) When the epithelial cell population neared confluence any residual feeder cells were removed by a brief incubation with an EDTA solution in order to aid a complete merging of colonies without any interspersed 3T3 fibroblasts. At one day post-confluence the cultures were rinsed and incubated with Dispase II (Boehringer-Mannheim) (2.5 mg ml⁻¹ in culture medium) to detach the cells as an intact sheet. The cell sheet was carefully rinsed with medium without disturbing its orientation and a sterile circular disc of silicone membrane (Silastic, Dow Corning, New Jersey) was placed on top of it.

A semicircular incision was made through the dorsal skin of the mouse and the flap was opened, leaving the muscle fascia exposed. The Silastic membrane and cell sheet was turned over and placed on the muscle fascia such that the basal cell layer of the cell sheet faced upward and the Silastic membrane contacted the muscle fascia. The skin flap was then folded back in place over the cell sheet so as to place the basal surface of the cultured cell sheet in contact with the underside of the mouse dermis, and the incision was closed with surgical staples (Ligacips, Ethicon, Inc., New Jersey).

Grafts were harvested after seven days, fixed in methanol/Carnoy's fixative (see Lindberg & Rheinwald, 1989), embedded in paraffin, and sectioned for hematoxylin-eosin (H & E) staining and immunostaining. 5 micron sections of grafts were stained by the avidin-biotin complex (ABC) peroxidase technique (Vector Laboratories, Burlingame, CA) or by indirect immunofluorescence, as described elsewhere (Lindberg & Rheinwald, 1989), using the following antibodies: CEP, a rabbit antisera specific for involucrin (Rice & Green, 1979); AKH1/8959, a rabbit antisera specific for filaggrin (Dale et al, 1985); and A53-B/A2, a mouse monoclonal antibody specific for keratin 19 (Karsten et al., 1985), purchased as antibody K19, 19.1 from ICN Biomedicals, Lisle, Illinois.

Acknowledgements

We especially thank Paul Kaplan for providing retroviral vectors and assistance in learning the defective retroviral transduction technique. John Wagner and Deborah Damon showed us how to perform and interpret the PC12 assay. We thank Drs. D. Rifkin and Y. Sato of N.Y.U. Medical School for providing anti-bFGF antisera, Dr. R. Rice of the Harvard School of Public Health for providing anti-involucrin antisera, and Dr. B. Dale of the University of Washington for providing anti-filaggrin antisera. We are
very grateful to Dr T. Kiropes of Ethicon Corp. for bFGF, Drs C. George-Nascimento and P. Valenzuela of Chiron Corp. for EGF and TGF-alpha, and Dr T. Maciag of the American Red Cross, Holland Labs for aFGF. Ms Therese O’Connell and Ms Beatrice Zenzie provided expert technical advice and assistance with keratinocyte and mesothelial cultures. This research was supported by grants from the National Foundation for Cancer Research, the National Cancer Institute, and a Faculty Research Award from the American Cancer Society to J.G.R., and by a program project grant from the National Cancer Institute to Dr A.B. Pardee.

References