Epidermal Cell Cultures from Involved Skin of Patients with Mammary and Extramammary Paget’s Disease

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Received for publication April 14, 1997

Summary: In involved epidermis, Paget cells are completely enclosed by the surrounding keratinocytes, which appear to be intact and unaltered. It is possible that the surrounding keratinocytes inhibit Paget cell proliferation. Accordingly, Paget cells might proliferate differently when cultured as an epidermal cell suspension. In this study, primary monolayer cultures of epithelial cells from involved epidermis of patients with mammary and extramammary Paget’s disease were carried out to investigate whether Paget cells proliferate in the same manner as other malignant cells. Skin samples were obtained from one patient with mammary Paget’s disease, and from 2 patients with extramammary Paget’s disease. The epidermis was separated from the dermis with dispase, and epidermal cell suspensions were obtained with ethylenediamine tetraacetate and trypsin. A commercially available serum-free media, Keratinocyte-SFM, was used. Epithelial monolayers from the involved skin could be maintained for approximately 45 days, while keratinocytes from normal skin were maintained for approximately 35 days. The mechanism for the longer survival of the mixed cell culture of keratinocytes and Paget cells is not known. Permanent cell lines were not developed from these primary cultures. Paget cells could not be distinguished from keratinocytes by phase-contrast microscopy. The proportion of carcinoembryonic antigen (CEA) positive cells in the culture did not increase, but instead decreased. In certain areas of the dish, the CEA positive cells proliferated and accumulated like mushrooms. However, at the periphery of the dish, the Paget cells identified by immunostaining for CEA were dispersed and not clustered. These findings indicate that the influence of keratinocytes on Paget cells also occurs in cultured cells, which may explain why Paget cells survive longer than keratinocytes. In conclusion, the Paget cells in the involved epidermis do not proliferate like other malignant cells.

Key words Paget cell, cell culture, cell cycle, CEA

Introduction

DNA histograms of Paget cells from the involved epidermis in mammary and extramammary Paget’s disease were similar to the histograms of normal...
keratinocytes (Mori et al. 1993a, b, 1994, 1995). Mammary Paget’s disease is probably a specialized form of ductal carcinoma that arises in the main excretory ducts of the breast and extends to involve the skin of the nipple and areola. However, the shapes of the cells in the epidermis are not very atypical and mitoses are rarely observed. In the involved epidermis, the Paget cells are completely enclosed by the surrounding keratinocytes, which appear to be intact and unaltered. From these observations, it is possible that the surrounding keratinocytes inhibit the proliferation of the Paget cells, but the Paget cells do not affect the keratinocytes. Accordingly, Paget cells cultured as epidermal suspensions, may proliferate differently because the influence of the surrounding keratinocytes would not be present.

In the present study, epithelial monolayer cultures from involved epidermis of mammary and extramammary Paget’s disease were performed in low Ca\textsuperscript{++} medium to prevent overrunning by fibroblasts and to confirm whether Paget cells exhibit different proliferative activity in vitro.

Materials and Methods

Skin samples were obtained from one patient with mammary Paget’s disease, and from 2 patients with extramammary Paget’s disease. Case 1, a 71-year-old woman, had a 3-year history of a progressively enlarging erythematous lesion on the right breast that was 53×44 mm in size and involved the nipple and areola. A biopsy of the nipple was taken, and, following the diagnosis of Paget’s disease, a modified mastectomy was performed. Case 2, a 75-year-old man, complained of a slowly enlarging, oozing lesion of 6 years’ duration on the left side of the scrotum and left groin that was 40×50 mm in size. Case 3, a 73-year-old man, had developed an erythematous eruption on the left side of the scrotum and penis shaft that was 30×40 mm in size. Cases 2 and 3 did not show dermal invasion, metastases of regional lymph nodes or underlying carcinomas. Normal tissue was taken from the left thigh as a control.

Cell culture

The skin specimens were cut into small pieces (approx. 1×1 cm) and soaked at 4 °C for 24 hs in 1,000 units/ml dispase (Godo Syusei, Tokyo, Japan) in phosphate buffered saline (PBS, pH 7.3). Epidermal sheets were separated from the dermis with forceps, rinsed with PBS, treated with a mixture of 0.1% ethylenediamine tetraacetate (EDTA) and 0.25% trypsin at 37 °C for 5 min and gently pipetted. The resulting cell suspensions of Keratinocyte-SFM (Gibco BRL, Gaithersburg, MD) were filtered through a fine mesh nylon net (0.2 mm pore size), plated on collagen-coated dishes (Rat tail collagen, Type I) (Collaborative Biochemical Products, Bedford, MA), and incubated at 37 °C in a humidified atmosphere of air containing 5% CO\textsubscript{2}. The cultures were observed daily for evidence of growth and the medium was changed at least twice a week. Confluent monolayers of tumor cells and normal cells grown in T-7 Falcon flasks were dispersed with trypsin. At each passage the cells were diluted 1:1. The 2nd- and 7th-passage epidermal cells from all subjects were diluted 1:1.
In one flask the media was changed to Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) for culturing in high calcium. Another was cultured in Keratinocyte-SFM.

Immunofluorescence microscopy

The cells grown on coverslips were fixed with methanol at -20°C for 10 min, and were incubated successively with PBS containing 1% bovine serum albumin for 30 min to reduce background staining. Thereafter, they were incubated with anti-human carcinoembryonic antigen (CEA) (DAKO, Copenhagen, Denmark) for 20 min at room temperature, followed by washing in PBS. They were then incubated with fluorescein isothiocyanate-labeled anti-rabbit IgG goat serum (DAKO, Copenhagen, Denmark) for 60 min at room temperature. After rinsing with PBS, they were mounted in glycerol-PBS and viewed under a standard microscope equipped for epifluorescence.

Quantitation of CEA positive cells was carried out on cultured cells seeded on coverslips. After subculture, 100 cells were counted in five different fields.

Immunohistochemical staining for CEA in formalin-fixed paraffin-embedded biopsy sections was carried out, as described previously (Mori et al. 1989). Quantitation of CEA positive cells was done by counting 100 epidermal cells on the sections in five different fields.

Results

Cell growth in a monolayer

The cells grew as a monolayer in Keratinocyte-SFM, rapidly becoming confluent but without stratification. Individual cells had a polygonal shape with a distinct intercellular space, giving the cell sheet a paving stone appearance when confluent. Many round cells were also observed floating in the medium. The morphology of 1st-passage epidermal cells is shown in Fig. 1. With phase-contrast microscopy, Paget cells could not be distinguished from epidermal keratinocytes. An outgrowth of fibroblasts was never observed. The cells were subcultured 13 times (48 days) in case 1, 9 times (41 days) in case 2, and 12 times (50 days) in case 3. Normal keratinocytes could be passaged an average of 9 times (36 days). In each case, the size of the cells increased with the number of subcultures.

Second-passage epidermal cells cultured in 10% FCS/DMEM from all subjects survived with stratification for only a few days and then they were overrun by fibroblasts and died. Seventh-passage cells were also killed by fibroblasts when cultured in a high calcium medium.

Fig. 1. First-passage epidermal cells observed with a phase-contrast microscope, 8 days after plating.
**Immunofluorescence microscopy**

In the cell culture, the CEA positive cells were dispersed, and not clustered at the periphery of the dish. However, the cells were somewhat extruded from the keratinocytes (Fig. 2). In contrast, the positive cells at the center were stratified like a mushroom and also seemed to be extruded from the keratinocytes (Fig. 3).

![Fig. 2. Immunofluorescence staining of CEA in cells at the periphery of the culture dishes. The CEA positive cells were dispersed, and seemed to be somewhat extruded from the keratinocytes.](image)

![Fig. 3. CEA staining of cells in the center of the dishes. The CEA positive cells were stratified like a mushroom and seemed to be extruded from the keratinocytes.](image)

The proportion of CEA positive cells decreased with the number of subcultures. At the 2nd-passage, the percentages of CEA positive cells were 6.91±1.14 in case 1, 6.74±1.28 in case 2, and 6.04±1.12 in case 3. At the 7th-passage, the percentages were 5.6±1.64 in case 1, 2.2±1.79 in case 2, and 5.2±1.45 in case 3.

For the biopsy sections, the proportions of CEA positive cells were 28.6±12.0 in case 1, 20.6±2.30 in case 2, 20.0±7.68 in case 3.

**Discussion**

While keratinocytes are not neoplastic, Paget cells have some phenotypes of neoplastic cells. Accordingly, it was anticipated that the keratinocytes would die prior to the Paget cells, and only the Paget cells would survive in the cell culture, because the inhibitory effect of the surrounding keratinocytes on the Paget cells would be removed. However, the epithelial monolayers could not be maintained for more than 45 days. Even the Paget cells from the involved epidermis of mammary Paget's disease, which should have originated from breast cancer, did not actively proliferate. Consequently, the proliferative potency of the Paget cells was similar to that for the keratinocytes in this system. In the epithelial monolayer culture, Paget cells were cultured with the keratinocytes. The Paget cells identified by staining for CEA were dispersed and not clustered at the periphery of the dishes, and they seemed to be somewhat extruded from the keratinocytes. However, at the center of the dish, the Paget cells were stratified like a
mushroom. The inhibitory influence of the keratinocytes on the Paget cells might remain in the culture, the same as in vivo, which could be one reason why the Paget cells were not maintained. In a previous study, the cell cycle of the Paget cells was measured, and the DNA histograms of the Paget cells from the involved epidermis in mammary and extramammary Paget’s disease (Mori et al. 1993a, b, 1994, 1995) were similar to the histograms of normal keratinocytes. However, the DNA histograms were determined in epidermal cell suspensions, but not with only Paget cells. Accordingly, it is possible that the Paget cells were suppressed by the keratinocytes in this experiment. This would explain why the DNA histograms of the Paget cells from the epidermis were similar to the normal ploidy.

The proportion of CEA positive cells among the epithelial cells did not increase with the number of subcultures. Two reasons must be considered for this phenomenon; 1) the number of Paget cells decreased, and 2) the Paget cells ceased to produce CEA. It is also possible that the decreased percentage of CEA positive cells is due to the low Ca++ medium. Thus, the epidermal cell suspensions were cultured in a high Ca++ medium, which seemed to be a better medium for the neoplastic cells. However, the Paget cells were killed by an overgrowth of fibroblasts. Growth in a culture of normal human epithelial cells has traditionally been very difficult, due both to fibroblastic overgrowth and to the inadequacy of media. Further investigations with new approaches that prevent fibroblastic overgrowth in the high Ca++ medium are needed.

This is the first report of a short culture of Paget cells, although the Paget cells were cultured concomitantly with keratinocytes. To further characterize the Paget cells, it is necessary to establish a culture with only Paget cells in future investigations.

References


