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The Effect of Partially Hydrolyzed Casein on the Growth of Human Skin Diploid Cells *in Vitro*[1] (40388)

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Casein is a phosphoprotein, precipitated from milk by dilute acid. Partially hydrolyzed casein (PHC) was prepared by hydrolyzing casein with trypsin at 46° for different periods of time to obtain varying amino nitrogen/total nitrogen ratios. This modified casein was thought to have wound healing properties in guinea pigs in preliminary experiments at Kraft Co. (unpublished results). The use of PHC in the local treatment of burns was found to accelerate burn healing (1–3). However, the mechanism of how PHC accelerates burn healing is still not well understood. Curtis, Brewer and Rose (1, 2) reasoned that PHC might prevent the loss of tissue protein by the formation of a type of sheltering film in burn treatment. Fleischer (3) speculated that the amino acids of PHC would supply the local nutritional requirements of the damaged cells in burn patients. Serum albumin and polypeptides have been reported to have growth promoting effects on cell growth *in vitro* (4–7).

The experiments in this report were performed in an attempt to investigate whether PHC can stimulate multiplication of human skin diploid cells *in vitro*. This system might be used as a model to study the mode of action of the burn healing process and possibly supplant the use of intact animals for measuring wound repair activity of PHC.

Materials and methods. Chemicals. Amino acids, vitamins and newborn calf serum were purchased either from International Scientific Industries, Inc., Cary, IL, or Grand Island Biological Co., Grand Island, NY; trypsin (1:250) from Difco Lab, Detroit, MI; *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; ethylene-diaminetetraacetic acid (EDTA) from Eastman Kodak Co., Rochester, NY and various forms of partially hydrolyzed caseins sterilized with ethylene oxide were generous gifts from Kraft, Inc., Research and Development, Glenview, IL.

Cell culture. Human skin diploid cells (prepuce cells) were established in our laboratory. The cells were prepared from tissue mincings of fresh foreskin of newborn babies according to the procedures of Anderson *et al.* (8) and Sandok *et al.* (9). A number of serial passages of the primary cells were made to establish a diploid cell monolayer culture. The cells were cultivated in 150 cm^2 polystyrene culture flasks (Corning Glass Works, Corning, NY) at 37° using Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) (10). The medium was buffered with 20 m*M* HEPES (11).

The cell monolayer was subcultured every 4–6 days. The cells were removed from the flask by discarding the growth medium, washing the cells three times with 7 ml of Hanks' balanced salt solution (BSS) (12) without calcium and magnesium salts (GKNP) and finally releasing the cells in 2 ml of 0.05% trypsin/0.05% EDTA in GKNP after 2–3 min of gentle shaking at room temperature. The cells were subcultured at a ratio of 3:1. Fresh medium was added to the cells and incubated at 37°. Cells were stored in fresh medium containing 15% glycerol at −80° in a Revco freezer or liquid nitrogen in vials or ampules containing 5×10^6 cells/ml until used.

Human kidney cells (SWINK) were also screened for growth promoting activity of casein. The cells were obtained from Dr. I. Tribby of the Abbott Laboratories and were cultivated in medium 199 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum. Swine testes cells (ST-92) obtained from Dr. S. McGregor, University of Wisconsin, Madison, and rabbit epidermal cells (SflEp) obtained from Dr. H. Fieldsteel, Stanford Research Institute, Menlo Park, CA, were also

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cultivated in Eagle's MEM (10) supplemented with 10% fetal calf serum.

Growth of prepuce and other cells in the presence of PHC. Prepuce cells were cultivated in Eagle's minimum essential medium containing 0% (MEM_0), 4% (MEM_4) or 10% (MEM_{10}) newborn calf serum in the presence of 0.4 and 1.6 mg/ml PHC. The medium was buffered with 20 m*M* HEPES and supplemented with 100 μg/ml of streptomycin and 100 units/ml of penicillin. When the effect of PHC was studied on cells cultivated in MEM_0 or MEM_4, the cells were cultivated in MEM_{10} initially for 24 hr. The medium was discarded and cells rinsed with BSS. Fresh medium containing PHC was added to the cells. The experiments were carried out in 25 cm^2 polystyrene cell culture flasks (Corning) at an initial cell density of 2.0–3.0 × 10^5 cells/flask containing 4 ml of medium. The cells were incubated at 37° for up to 7–8 days and enumerated at varying intervals of time by removing cells from the flask with trypsin. Cells were counted with the aid of a Coulter counter. A minimum of two independent experiments were performed. Each treatment was carried out in three flasks and triplicate counts were made from each flask.

The SWINK, ST-92 and SflEp cells were tested with PHC in the same manner as prepuce cells.

Results. Prepuce cells after trypsinization lose some viability, therefore to interpret amounts of growth stimulation, the cell population found after 24 hr incubation was used as a baseline control to interpret the data.

The graph presented in Fig. 1 shows the effect of partially hydrolyzed casein I (PHC I) (amino N/total N ratio, 0.09) on the growth of prepuce cells cultivated in MEM_{10} medium. The addition of PHC I to the medium did not significantly change the cell growth compared to the control during the first 3 days of incubation. On day 5, in the presence of 0.4 or 1.6 mg/ml PHC I, increases were observed of about 50% and 113%, respectively, in cell number over the control cells. The number of prepuce cells cultivated in medium supplemented with 1.6 mg/ml PHC I began to decrease after day 5, whereas control cells and to a much lesser degree, cells treated with 0.4 mg/ml PHC I, continued to increase in cell number. Nevertheless, numbers of cells supplemented with 0.4 or 1.6 mg/ml PHC I were still 35% and 64%, respectively, higher than that of control cells on day 7.

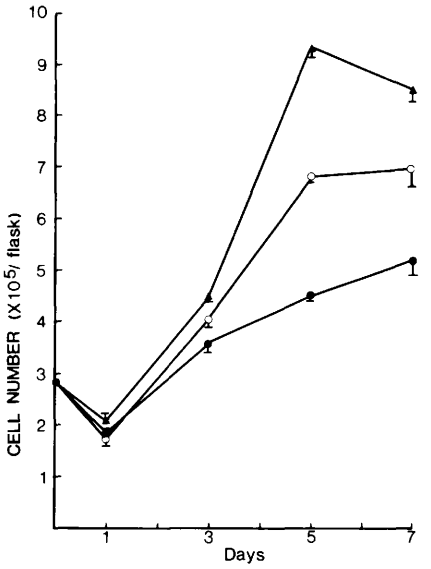

FIG. 1. Effect of partially hydrolyzed casein I (PHC I, amino N/total N ratio of 0.09) in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) on growth of prepuce cells. An initial inoculum of 2.8 × 10^5 prepuce cells in 4 ml of MEM_{10} supplemented with PHC I was placed in 25 cm^2 polystyrene cell culture flasks and incubated at 37° for 7 days. Cells were enumerated with a Coulter counter at varying intervals of time after incubation. Each point represents the mean ± SEM of triplicate counts from three flasks. The results are typical of six independent experiments. ●——●, MEM_{10}; ○——○, 0.4 mg/ml PHC I in MEM_{10}; ▲——▲, 1.6 mg/ml PHC I in MEM_{10}.

The growth stimulating activity of 1.6 mg/ml PHC I on prepuce cells in medium containing 0 or 4% newborn calf serum was similar to that in medium containing 10% newborn calf serum (Fig. 2). Numbers of cells cultivated in MEM_4 supplemented with 1.6 mg/ml PHC I showed increases of 41%, 47% and 31% over that of cells grown in MEM_4 alone on day 3, 6 and 8, respectively.

Cells grown in medium without serum and no PHC I supplement did not multiply over the entire incubation period. Addition of 1.6 mg/ml PHC I to medium containing no serum resulted in 2.5-fold of multiplication after 8 days of incubation.

Figure 3 shows the effect of partially hydrolyzed casein II (PHC II, amino N/total N

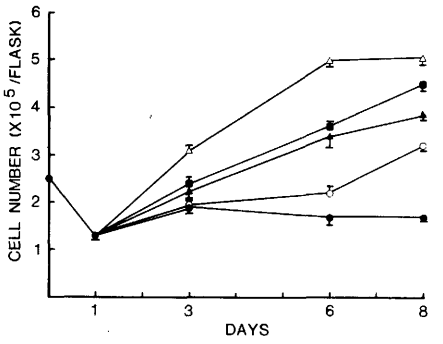

FIG. 2. Effect of partially hydrolyzed casein I (PHC I) in Eagle's minimum essential medium supplemented with 0% (MEM_0) or 4% (MEM_4) newborn calf serum on growth of prepuce cells. An initial inoculum of 2.5×10^5 cells in 4 ml of MEM_{10} was placed in 25 cm^2 polystyrene cell culture flasks. After 24 hr incubation at 37° the medium was discarded and the cells rinsed with Hanks' balanced solution (BSS). MEM_0 or MEM_4 supplemented with or without PHC I was added to the cells and incubated at 37° for 7 more days. Unsupplemented MEM_0 and MEM_4 media were used as controls. Each point represents the mean ± SEM of triplicate cell counts from three flasks. The results are typical of at least two independent experiments. ■——■, cells grown in MEM_{10}; ●——●, cells grown in MEM_0; ○——○, cells grown in MEM_0 + 1.6 mg/ml PHC I; ▲——▲, cells grown in MEM_4; △——△, cells grown in MEM_4 + 1.6 mg/ml PHC I.

ratio of 0.36) on the growth of prepuce cells cultivated in MEM_{10}. There was little effect of PHC II on cell growth. Partially hydrolyzed casein III (PHC III) having a higher amino N/total N ratio of 0.70 had a similar effect as PHC II on growth of prepuce cells cultivated in MEM_{10}, as one might expect.

The effect of PHC I on growth of human kidney cells (SWINK) is shown in Table I. There is a suggestion that 1.6 mg/ml of PHC I appears to stimulate growth of the cells after 120–168 hr of cultivation. Growth-stimulation of SWINK cells was less than prepuce cells in the presence of 1.6 mg/ml PHC.

Swine testis and rabbit epidermal cells cultivated in MEM medium with 10% fetal calf serum did not show any changes in growth in the presence of 0.4–1.6 mg/ml PHC I.

Discussion. The results of the experiments illustrated in Fig. 1 show that PHC I enhanced the growth of prepuce cells cultivated in MEM_{10}. It was found that prepuce cells could multiply to some extent in medium without serum supplement when PHC I was added to the medium (Fig. 2). The growth curve in Fig. 2 also shows that cell populations in medium supplemented with 4% newborn calf serum and PHC I was higher than that of cells grown in medium containing 10% newborn calf serum alone. These observations suggest that PHC I contains growth-stimulating activity for growth of prepuce cells which resembles activity of newborn calf serum.

Dulak and Temin (4) and Smith and Temin (5) have isolated and purified polypeptides with growth-stimulating activity for

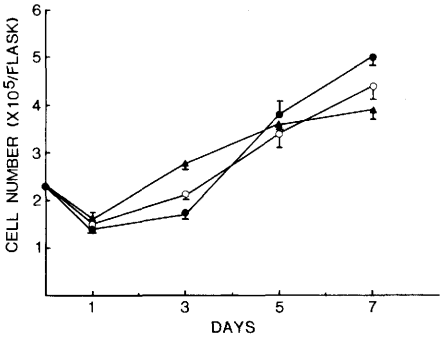

FIG. 3. Effect of partially hydrolyzed casein II (PHC II, amino N/total N ratio of 0.36) in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) on growth of prepuce cells. An initial inoculum of 2.3×10^5 prepuce cells in 4 ml of MEM_{10} containing PHC II was placed in 25 cm^2 polystyrene cell culture flasks and incubated at 37° for 7 days. Each point represents the mean ± SEM of triplicate counts from three flasks. The results are typical of two independent experiments. ●——●, control; ○——○, 0.4 mg/ml PHC II; ▲——▲, 1.6 mg/ml PHC II.

TABLE I. EFFECT OF VARYING AMOUNTS OF CASEIN HYDROLYSATE I (PARTIALLY HYDROLYZED) ON HUMAN KIDNEY CELLS (SWINK).

Casein supplement (mg/ml)	Hours incubation—37°				
	0	24	72	120	168
0.0	3.7[a]	3.1	8.0	6.9	7.2
0.4	5.6	4.1	7.7	7.2	9.3
0.8	3.3	2.2	7.9	8.5	10.0
1.6	4.0	3.3	8.7	9.3	12.0
3.2	4.5	2.5	8.2	8.1	11.0

[a] Cell count/flask × 10^5. Cells were cultivated in medium 199 (Grand Island Biologicals) supplemented with 10% fetal calf serum. The results are typical of two independent experiments. Three samples from each of three flasks were counted in triplicate. Counts were performed on a Coulter counter or Biophysics cytograph.

chicken and rat embryo fibroblasts. Spieker-Polet and Polet (6, 7) have reported that serum albumin stimulated growth of activated human lymphocytes. They have further shown that the growth-promoting activity of albumin was completely abolished by pepsin digestion. Our results support this finding. When casein was hydrolyzed for extended periods of time with trypsin, a product having amino N/total N ratio of 0.36 or 0.70 was obtained, destroying the growth-stimulating activity for prepuce cells (Fig. 3). There is a suggestion that the growth-stimulating activity of casein is attributed to the casein itself as well as presence of optimum amino acid concentration and/or polypeptide fractions. Comparison of amino acid and polypeptide analyses of trypsinized casein fractions could further quantitate the optimal amounts of these materials for cell growth.

PHC I could be used to replace serum in cell culture studies. Only prepuce cells, of the four cells screened, appear to show consistent patterns of increase of growth in the presence of PHC I. PHC I did not stimulate growth of human kidney, swine testis and rabbit epidermal cells under the experimental conditions used. It appears that cells that grow slowly *in vitro* may be more easily stimulated by PHC I than fast growing cells.

An assay for PHC I in prepuce cell cultures could be eventually used to standardize doses of sterile casein for burn or other wound healing treatments, substituting in part animal efficacy tests.

Summary. The effect of various partially hydrolyzed caseins (PHC) on the growth of human skin diploid (prepuce) cells cultivated *in vitro* has been investigated. The results indicate that PHC I (amino N/total N ratio of 0.09) contains growth-stimulating activity for prepuce cells in the absence and presence of serum. This activity of PHC I was abolished when casein was further hydrolyzed with trypsin (amino N/total N ratio of 0.36 or 0.70). It is suggested that the growth-stimulating activity of casein is attributed to the casein itself or the presence of optimal amounts of amino acids and/or polypeptide fractions. Human kidney (SWINK), swine testis (ST-92) and rabbit epidermal cells (SflEp) showed little or no growth stimulation in any of the concentrations of PHC I tested. A cell culture system to assay PHC I activity is now available which could eventually short-circuit the routine use of animal assays to test burn or other wound healing cell activity. PHC I appears to substitute for serum in growth of prepuce cells *in vitro* so that another serum-free medium is now available for certain types of experiments.

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