A growth-modifying factor was detected in the culture media derived from flask cultures of three established lines of human malignant origin. The growth-modifying factor migrated electrophoretically like prealbumin, was antigenic, and showed hyaluronidase activity. This protein stimulated fibroblastic growth at low concentrations but stopped mitosis and was cytotoxic to fibroblasts at high concentrations. Lines of identity in an immunodiffusion system suggested that a common antigen was being produced by carcinoma cells of divergent sources but was not produced by fibroblasts.

INTRODUCTION

Reports of work with conjoint tissue cultures of normal and neoplastic cells appear to offer conflicting evidence for both stimulatory and inhibitory effects on fibroblasts. Ludford and Barlow (11, 12) reported that the growth of fibroblasts was stimulated by diffusible agents derived from carcinoma cells but was inhibited by sarcoma cells. These findings were consistent with the observations of Kasuta et al. (9), who used parabiotic cultures. Ranadive and Bhide (13) concluded from studies with conjoint tissue cultures that the growth-stimulating activity of tumor cells is closely related to the phase of tumor progression and grade of malignancy. Foley et al. (3) also found that fibroblasts grew more rapidly in the presence of HeLa cells. In contrast, Smith and Cress (14) and Holmberg (7) extracted products from cancer cells and found them injurious to connective tissue elements. These extracts were toxic to fibroblasts at concentrations that had no effect on cancer cells. Hymes et al. (8) reported intermediate effects with a prealbumin component from the ascitic fluid of rats bearing a Walker 256 carcinoma. The inconsistency in these reports leaves room for further study, designed to characterize the nature of the extractable substance and to describe its effects on fibroblasts. The purpose of this study was to attempt: (a) to isolate and characterize a chemical substance which has a measurable effect on the growth and morphology of fibroblasts and (b) to unify the conflicting reports in the literature.

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MATERIALS AND METHODS

The established human cell lines used in this study included the KB line, derived from a human nasopharyngeal carcinoma; the CMP line, originating from an adenocarcinoma of the colon; and HeLa, a cervical carcinoma. The nonmalignant cell type used most frequently was a human embryonic skin fibroblast strain. For 1 set of experiments, an established line of human Amnion (Fernandes) was also used. The cells were maintained in either T-60 flasks or Rose multipurpose culture chambers in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. This serum showed no electrophoretically separable prealbumin component.

The GMF was harvested by collecting the culture medium from a confluent layer of cells incubated for 48 hr or longer in T-60 flasks. When the medium was to be introduced into fibroblast cultures, it was simply centrifuged to remove cellular debris and neutralized with dilute NaOH. If the factor was to be concentrated, the cells within the flasks were rinsed gently 3 times with Hanks’ balanced salt solution and then incubated for 48 hr in serum-free Eagle’s medium. The pooled medium was then centrifuged and back-dialyzed against Carbowax for 8 to 12 hr. For antigen preparation, the factor was precipitated with 40% ammonium sulfate from serum-free medium and then dialyzed overnight against balanced salt solution.

The polyacrylamide gels were prepared according to the procedure outlined by Davis (2). The best separation of the GMF was accomplished with 7.5% acrylamide and 0.184% N,N'-methylenebisacrylamide in a current of 50 ma for 45 mm. The bands were demonstrated by standard Amido schwarz staining procedures.

Anti-GMF serum was prepared in rabbits with an antigen collected from serum-free medium that had maintained viability of CMP cells for 48 hr or longer in T-60 flasks. When the medium was to be introduced into fibroblast cultures, it was simply centrifuged to remove cellular debris and neutralized with dilute NaOH. If the factor was to be concentrated, the cells within the flasks were rinsed gently 3 times with Hanks’ balanced salt solution and then incubated for 48 hr in serum-free Eagle’s medium. The pooled medium was then centrifuged and back-dialyzed against Carbowax for 8 to 12 hr. For antigen preparation, the factor was precipitated with 40% ammonium sulfate from serum-free medium and then dialyzed overnight against balanced salt solution.

The polyacrylamide gels were prepared according to the procedure outlined by Davis (2). The best separation of the GMF was accomplished with 7.5% acrylamide and 0.184% N,N'-methylenebisacrylamide in a current of 50 ma for 45 min. The bands were demonstrated by standard Amido schwarz staining procedures.

Anti-GMF serum was prepared in rabbits with an antigen collected from serum-free medium that had maintained viability of CMP cells for 48 hr. The antiserum was placed in a central well in an agar-filled Petri dish, while antigens from various media were placed in surrounding wells, according to procedures described by Crowle (1). The precipitin bands, which developed at room temperature over a period of 48 hr, were photographed with dark-ground illumination. The anti-human prealbumin serum was kindly furnished by Hyland Laboratories, Los Angeles, Calif.

A volume of 10 ml of whole blood was collected from a human volunteer and chilled for 2 hr. The overlying plasma
was carefully pipetted off, and chick embryonic extract was added to promote clotting of the upper buffy coat layer. When the clot was firm, this leukocyte-rich fraction was dissected away from the underlying erythrocyte fraction. The clot was then trimmed into 1- to 2-sq mm explants, which were placed in Rose multipurpose culture chambers. The fragments were held in place by a secondary clot comprised of 1 drop each of rooster plasma and embryonic extract. When the clots were firm, the chambers were filled with either complete control medium or medium that had sustained CMP cell growth for 48 hr. The outward migration of leukocytes formed a halo, which was recorded as a shadowgram after 72 hr by placing the chamber in a photographic enlarger. The areas of the original explant and of the outgrowth were measured from the enlarged print with a planimeter. Average outgrowth to explant area ratios were calculated.

Hyaluronidase activity was determined for commercial bovine testicular hyaluronidase and for GMF derived from CMP culture medium with the colorimetric method of Morgan and Elson, as described by Greiling (6). In each case, a condensation product of p-dimethylaminobenzaldehyde and disaccharide from digested hyaluronic acid showed an absorption characteristic with peaks at 545 and 582 nm, when measured with a Beckman Model DB recording spectrophotometer.

RESULTS

Cell-free culture media harvested from flasks of KB, CMP, and HeLa cells were found to be toxic to fibroblasts derived from human embryonic skin (Fig. 1). Although the control cultures (Fig. 1a) showed cellular confluence and mitotic activity, cultures from the same set, which were treated with CMP medium, contained cells showing cytoplasmic retraction, zonosis, and death (Fig. 1b). An analysis of the surviving fibroblast population, 48 hr after treatment with media from the 3 cell lines, was made with a Coulter electronic cell counter. The data are presented in Chart 1. While the cell counts suggested that the KB and CMP media were more toxic than the HeLa medium, the data were not corrected for variance in the cell numbers of the cell lines used to generate the GMF. Therefore, these data have only qualitative value.

Cell-free culture media, harvested from cultures of CMP, HeLa, and KB cells, were found to contain a prealbumin band in an acrylamide gel electrophoretic core, which was absent in aliquots of the same media that had not been used to sustain cell growth (Fig. 2). No such band could be detected from media that had supported the growth of human embryonic skin fibroblasts. Replicate experiments with serum-free media showed only the prealbumin band in the aliquot which had been on the established cell lines for a period of 48 hr.

With the Ouchterlony immunodiffusion techniques, it was observed that anti-GMF serum reacted with antigens from the culture medium which had supported the established lines of KB, CMP, HeLa, and Amnion cells. The precipitin lines from KB, CMP, and HeLa antigens were continuous, which suggested that their synthesized products contained immunologically similar stereoconfigurations. Although the Amnion cell line produced a reactive antigen, it did not show lines of identity with the others. No antigens that would react with the anti-GMF serum were found from either control medium or nutrient fluid harvested from fibroblast cultures (Fig. 3).

The anti-human prealbumin showed a positive reaction with electrophoretically separated human serum prealbumin, but it was not reactive with the antigen at the same concentration derived from 48-hr cultures of CMP cells maintained on serum-free medium (Fig. 4). Therefore, GMF was considered to be electrophoretically similar to human serum prealbumin but was immunologically different.

The synthetic product from the 3 malignant cell lines was nondialyzable, showed a positive reaction for peptides and proteins with both biuret and Lowry tests, was precipitable with both alcohol and trichloroacetic acid, and showed an absorption maximum at 280 nm. In an effort to make a comparison of this factor with a rat "prealbumin" fraction described by Hymes et al. (8), the GMF was discovered to be heat labile. The outgrowth to explant area ratio of human buffy coat cells maintained in control medium supplemented with 10% fetal calf serum was 1.86 ± 0.48. A ratio of 0.97 ± 0.35 reflected an inhibitory effect on the migratory rate of human leukocytes when bathed in CMP medium. When an aliquot of the same medium was heated to 90° for 5 min, the ratio increased to 2.30 ± 0.42. When GMF was incubated with hyaluronic acid, disaccharide breakdown products detected with the colorimetric reaction of Morgan and Elson revealed a weak hyaluronidase activity. Collectively, these data strongly suggested that the synthetic product was protein.

The prealbumin component extracted from pooled serum-free media that had supported the growth of CMP cells for 48 hr was collected in large quantities and back-dialyzed against Carbowax. The protein concentration was then evaluated by the Lowry method and was diluted to form stock solutions ranging from 0 to 20 mg of protein/ml of complete Eagle's medium supplemented with 10% fetal calf serum. These media were added to cultures of human skin to study the effect on their morphology and activity.

The data in Table 1 indicate that the mitotic activity of
<table>
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<th>Total no. of cells scored</th>
<th>No. of mitotic figures</th>
<th>% of mitosis</th>
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<td>0 (Control)</td>
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Chart 2. Fibroblast survival following 48 hr in culture medium containing various concentrations of GMF harvested from CMP medium. Numbers along the ordinate are to be multiplied by $10^5$ to obtain the correct value. CONT, control.

The data in Table 1 and Chart 2 indicate that fibroblasts can be either stimulated or inhibited by varying the concentration of the GMF. This phenomenon could account for conflicting reports of the effects of tumor extracts on connective tissue elements. The response of fibroblasts appears to be due to quantitative, not qualitative, variations in the extract.

REFERENCES


Fig. 1. a, a confluent layer of human skin fibroblasts in control medium; b, identical culture of human skin fibroblasts after 24 hr in CMP medium.

Fig. 2. Electrophoretic distribution of fetal calf serum components from control medium (Cont) and CMP medium (CMP). The band indicated by the arrow is considered to be the GMF.

Fig. 3. Anti-GMF serum precipitin reactions with different culture media. No reaction occurred with control medium (Cont) and that from fibroblast cultures (Fibro). Discontinuous lines were formed with Amnion medium. A line of identity indicated the presence of an antigen common to KB, CMP, and HeLa cells.

Fig. 4. Anti-human prealbumin serum (As) reacted strongly with human prealbumin (Pre Alb) but not with GMF at the same protein concentration.
A Growth-modifying Factor from Cell Lines of Human Malignant Origin

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