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EFFECT OF LACTOFERRIN ON THE GROWTH OF A HUMAN COLON ADENOCARCINOMA CELL LINE— COMPARISON WITH TRANSFERRIN

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SUMMARY

Lactoferrin was examined for its effect on the growth of a human colon adenocarcinoma cell line (HT 29) in culture and its action was compared to that produced by transferrin and two different iron solutions (ferrous sulfate and ferric chloride). When transferrin was replaced by either iron solutions the cell grew in proportion to the quantity added and the maximal effect obtained was identical to that produced by transferrin alone. When transferrin was replaced by lactoferrin the cells were unable to proliferate for a long time. However, in the presence of low-concentration iron solutions, lactoferrin stimulated the cell growth, and the effect was more pronounced with the ferric chloride solution.

Key words: lactoferrin; cell growth; serum-free medium; transferrin.

INTRODUCTION

Pichon et al. (1) using an adenocarcinoma cell line (HT 29), described a completely defined serum-free minimal medium that promotes growth comparable to that obtained in serum containing medium while maintaining the differentiated characteristics of the cell. This medium contains selenium, epidermal growth factor, and transferrin (Tf). Transferrin was first demonstrated by Hayashi and Sato (2) to be essential for the growth of HeLa cells in serum-free medium and is now used for the growth of other cell lines in synthetic medium (3,4). It was suggested further that the Tf dependence of maximal cell growth in culture was mediated through the expression of a Tf receptor that could play an essential role in transport of Fe across the cell membrane (5). More recently, it was demonstrated that monoclonal antibodies directed against the human Tf receptor inhibited the growth of human melanoma cells in nude mice (6) and may block the growth of a human T leukemic cell line (CCRF-CEM) in vitro (7).

Transferrin presents structural and functional homology with lactoferrin (Lf), another iron-binding glycoprotein present in epithelial cells of the exocrine glands (8), as well as in the specific granules of polymorphic granulocytes (9). Both molecules have the same molecular weight (about 75 000), consist of a single polypeptide chain to which two carbohydrate groups are attached, and are able to bind two iron Fe III ions tightly in two specific iron binding sites (10). The essential features of the metal binding sites of the two proteins are similar, and six tyrosine residues as well as four histidine residues are involved in the binding of the two ferric ions (11,12). In addition, the alternative occurrence of Lf or Tf as the dominating iron-chelating protein in the milk of different species (13) suggests a common biological effect of the iron-linking part of the two molecules. However, Lf differs from Tf in its ability to retain iron at low pH (14), and some dissimilarity in the behavior of the two iron-binding sites has been demonstrated (15).

The iron-binding properties of Lf led us to study the effect of the protein on cell growth in culture, and in this paper we report the effect of Lf on the growth of an adenocarcinoma cell line (HT 29) and compare its action to the stimulating effect of Tf.

MATERIALS AND METHODS

Materials. Insulin and Tf (substantially iron free) were purchased from Sigma, St. Louis, MO.

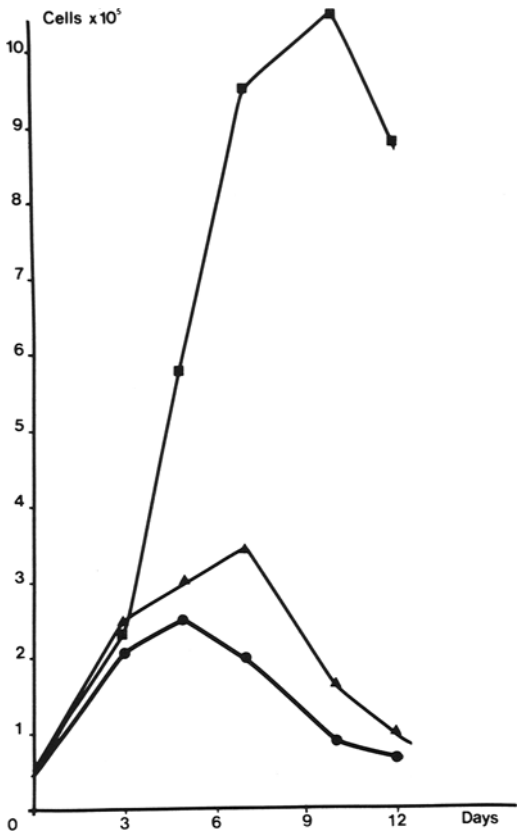

FIG. 1. Effect of Lf or Tf on the growth curves of HT 29 cells. The cells were grown in minimal serum-free medium (already containing 0.45 μg ferrous sulfate/ml) supplemented with EGF and Se as described in Materials and Methods (●--●). Cells grown in the same medium supplemented with Lf 7 μg/ml (▲—▲); Cells grown in the same medium supplemented with Tf 7 μg/ml (■--■).

Dulbecco's modified minimum Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (PBS), Ham's F12 medium, fetal bovine serum (FBS), and trypsin were from GIBCO, Grand Island, NY. Human apo Lf (Lf) purified from human milk as described by Masson (13) was a gift of Dr. P. Masson (Brussels).

Culture conditions. HT 29 (a gift of Dr. Zweibaum, Paris) is a malignant cell line in culture originating from human colon (16). The cells were tested by biochemical procedure and found mycoplasm-free. HT 29 cells were grown routinely at 37° C in a humidified atmosphere of 95% air:5% CO_2. The culture medium was DMEM supplemented with 10% FBS and extemporaneously prepared. Cells were harvested with 0.05% trypsin/0.53 m*M* EDTA in PBS and plated in 75 cm^3 flasks for at least 48 h before being used.

Experimental procedure. For growth assays, HT 29 cells were washed with EDTA and trypsinized. The cells were harvested and diluted with an equal volume of 10% FBS-supplemented medium, centrifuged, and resuspended in the same medium. Then samples ($5 \cdot 10^4$ cells/1 ml) were plated in quadruplicate in 24-well Costar dishes. The next day, the medium was replaced by the serum-free synthetic medium. The "defined" serum-free medium used was a mixture of 1:1 Ham's F12 and DMEM media containing 15 m*M* HEPES, selenium (10 ng/ml), epidermal growth factor (EGF, 5 ng/ml), and Tf (7 μg/ml); the ferrous sulfate concentration in the medium was 0.45 μg/ml. This simplified system allows one to study the replacement of Tf in the culture medium by different solutions. Experiments required a change of medium every 2 to 3 d. This was done carefully, removing the medium from each plate and replacing it with fresh medium.

After trypsinization, the number of viable cells was determined by counting, every 2 or 3 d, the number of cells that excluded the trypan blue dye. Each experimental value was the average number of cells in four wells with a variation coefficient about 15%.

RESULTS

Comparative effect of Lf and Tf on cell growth. The first growth experiments were performed in

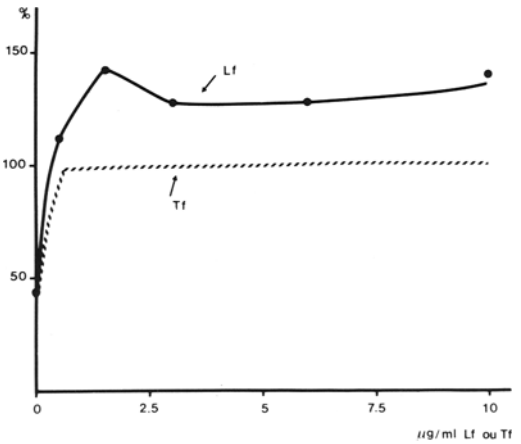

FIG. 2. Dose-effect growth curves of HT 29 cells in response to Lf or Tf. The cells were grown for 3 d in the minimal serum-free medium as delineated in Fig. 1 supplemented with different doses of Lf (●--●). The results are expressed as percentage of the control value obtained by adding Tf instead of Lf to the medium (---).

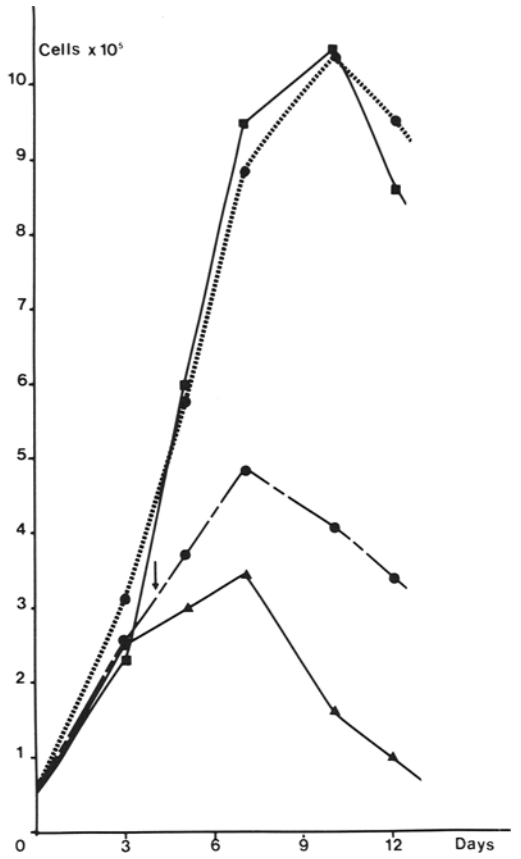

FIG. 3. Effect of different components on the growth curves of HT 29 cells. The cells were grown in the same serum-free medium as delineated in Fig. 1, supplemented either with Lf (▲—▲) or with Tf (■—■), or with Tf instead of Lf, 4 d later (*arrow*) (●——●), or with ferrous sulfate along at the dose of 3 μg/ml (●----●).

the minimal synthetic medium (supplemented with selenium and EGF) with Tf or LF added individually to cells at concentrations of 7 μg/ml. The growth response was followed for 12 d and compared to a control performed with the minimal medium alone.

Figure 1 shows that cells cultured in the presence of substantially iron-free Tf grew normally, as already described (1). Similar results were obtained when the cells were grown in the presence of the same Tf treated by extensive dialysis against 0.1 *M* citrate buffer at pH 2.3, as described in (15), to remove an eventual residual iron content (data not shown). By contrast, the growth curves obtained with Lf were quite different. During the first 3 d of growth the cells cultured in the presence of LF grew better than with Tf, whatever the concentration of protein used (Fig. 2). Thereafter, the cells proliferated at a slower rate before they decreased as did the cells cultured in the minimal medium alone (Fig. 1). In addition, it must be noted that when examined under a light microscope, cells cultured with Lf were larger than cells cultured with Tf and they exhibited a granular cytoplasmic structure.

Because Tf was a commercial preparation that might contain other essential serum products, such as insulin or triiodothyronine (T3) whose stimulating effect on growth cell was clearly established on different cell lines but not in HT 29 cells (1), cells were cultured in the presence of Lf supplemented with insulin (2 μg/ml) or with insulin (2 μg/ml) plus T3 (0.5 pmol/ml). The resulting growth curves showed an even lower

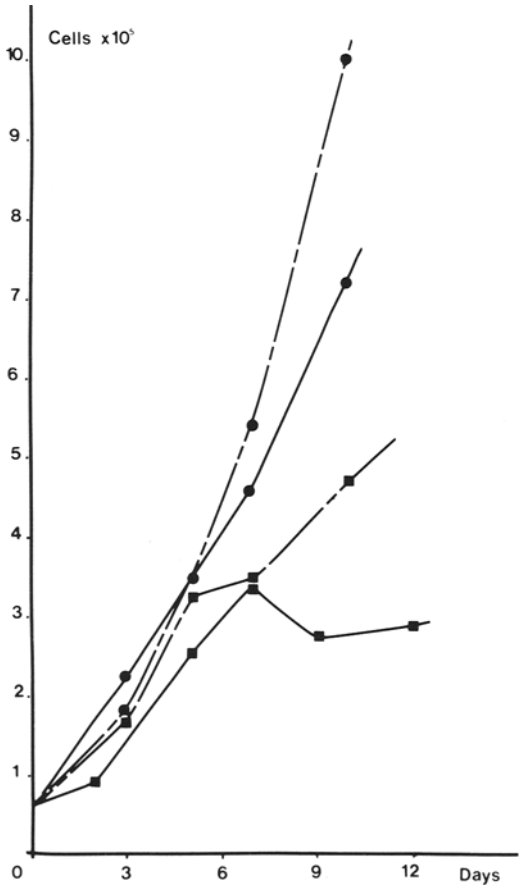

FIG. 4. Effect of different iron solutions on the growth curves of HT 29 cells. The cells were grown in the same serum-free medium as delineated in Fig. 1 supplemented either with ferrous sulfate 0.5 μg/ml (■——■), ferrous sulfate 3 μg/ml (●——●), ferric chloride 0.5 μg/ml (■—■), or ferric chloride 3 μg/ml (●—●).

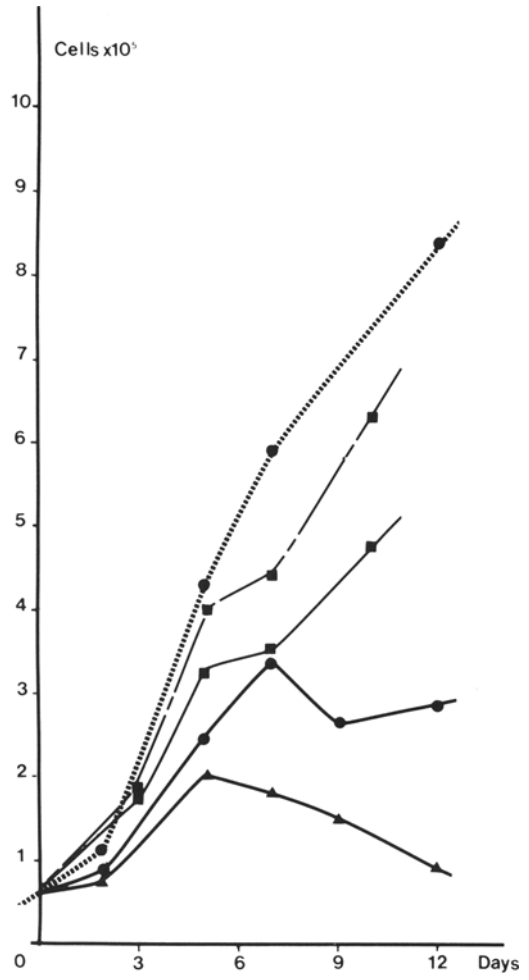

FIG. 5. Effect on the growth curves of HT 29 of Lf in the presence of low-concentration iron solutions. The cells were grown in the same serum-free medium as delineated in Fig. 1 supplemented with either Lf (7 μg/ml) alone (▲—▲), with ferric chloride 0.5 μg/ml (●—●), with Lf 7 μg/ml + ferric chloride 0.5 μg/ml (●---●), with ferrous sulfate 0.5 μg/ml (■—■), or with Lf 7 μg/ml + ferrous sulfate 0.5 μg/ml (■---■).

proliferation rate than that obtained with Lf alone (data not shown).

Growth stimulatory activity of ferrous sulfate and ferric chloride solutions. After adding 3 μg ferrous sulfate/ml to the minimal medium (already containing 0.45 μg ferrous sulfate/ml), the growth rate of the cells was similar to that of cells growing in the presence of Tf (Fig. 3). Identical results were obtained when Lf was added together with ferrous sulfate, demonstrating the lack of a toxic effect of lactoferrin itself on the epithelioma cell line. The cells cultured first in the presence of Lf for 5 d, then in minimal medium where transferrin had been substituted for lactoferrin, were able to proliferate more rapidly than the cells left in presence of Lf but never reached the proliferation rate of the cells continuously grown with Tf (Fig. 3).

Different iron solutions were examined for their effect on cell growth; the results are reported in Fig. 4. Growth-stimulatory activity of the solutions seems proportional to their iron concentration inasmuch as ferrous sulfate and ferric chloride solutions were more effective at the concentration of 3 μg/ml than at a concentration 10 times lower. In any case, the ferrous sulfate solution was more effective than the ferric chloride solution.

Growth stimulatory activity of Lf in the presence of low concentrations of ferrous sulfate or ferric chloride solutions. The growth responses of HT 29 cells to the addition of Lf to the minimal synthetic medium (already containing 0.45 μg ferrous sulfate/ml) supplemented with low-concentration iron solutions are given in Fig. 5. In both cases the iron-binding protein exerts a powerful stimulation on the cell growth, and the effects were more pronounced with the ferric chloride solution.

Dose effect of ferric chloride on the cells grown in the presence of Lf (7 μg/ml). As demonstrated in Fig. 6, Lf stimulates the cell growth in the presence of ferric chloride solutions at any concentration, but the more dramatic effect was obtained with a concentration of ferric chloride equal to 0.5 μg/ml.

DISCUSSION

The results of the present investigation illustrate several interesting aspects of cell growth:

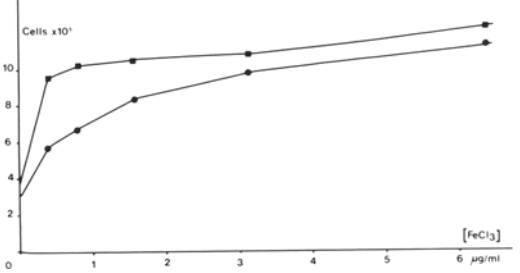

FIG. 6. Dose-effect growth curves of HT 29 cells in the presence or absence of Lf in response to ferric chloride. The cells were grown for 8 d in the minimal serum-free medium as delinated in Fig. 1 in the absence of Lf (●—●) or supplemented with Lf 7 μg/ml (■—■).

TABLE 1

EFFECT OF Lf AND Tf ON THE DISSOCIATION OF [^{125}I]Lf BOUND TO HT 29 CELLS[a]

Proteins Added in Competition	[^{125}I]Lactoferrin CPM Binding	
	Experiment 1[b]	Experiment 2[c]
—	692	2247
Tf (200 μg)	799	2025
Lf (200 μg)	290	1280

[a] All experiments were performed in duplicate.

[b] $6 \cdot 10^5$ Cells were incubated in DMEM medium supplemented with 0.1% BSA with 75 000 cpm of chloramine T. iodinated Lf.

[c] Same conditions as used in experiment 1 but with 10^6 cells and 200 000 cpm.

They provide conclusive evidence that Lf cannot be substituted for Tf to promote a long-term growth of HT 29 cells in the "defined" medium, medium containing 0.45 μg/ml ferrous sulfate. It is well known that ferrous salts are converted easily in ferric salts in aerobic conditions and that the effect is greatly enhanced in the presence of iron-binding proteins that have a strong affinity for ferric salts. However, in this medium, Lf was completely unable to allow cell proliferation whereas Tf permitted maximal cell growth (1). The initial iron saturation of the two proteins cannot explain the differences in the biological activities because all the experiments were performed with apo-Lf and the commercial, "substantially" iron-free Tf and apo-Tf gave exactly the same results. It is clear that, despite structural similarities, the two iron-binding proteins behave differently in the defined culture medium.

When the minimal "defined" medium was supplemented with 0.5 μg/ml ferrous sulfate or 0.5 μg/ml ferric chloride, which alone at this concentration do not support maximal cell growth, the addition of Lf greatly enhanced HT 29 cell growth. This is paradoxical inasmuch as the minimal defined medium already contained 0.45 μg/ml ferrous sulfate. It is possible that the defined medium was in fact devoid of iron salts because, as noted by Ham (17), the iron in the dry powdered medium may become oxidized and fail to redissolve and to pass through the sterilizing filter. Nevertheless, our data show clearly that if transferrin, but not lactoferrin, promotes the growth of HT 29 cells in the defined medium, lactoferrin may play the role of iron-carrier protein when the medium is supplemented by 0.5 μg/ml ferrous sulfate or ferric chloride. The more dramatic effect in the presence of the ferric solution could be because lactoferrin, like transferrin, can only bind ferric ions. These data are in agreement with the results obtained by Trowbridge and Lopez (7) who have shown recently that in some cases only ferric complexes could partially overcome the inhibitory effect of a monoclonal antibody directed against the transferrin receptor on CCRF-CEM cells grown in serum-supplemented medium.

The amount of protein has by itself no specific effect on cell proliferation because cells grown in the presence of an excess of ferric chloride (3 μg/ml) or ferrous sulfate (3 μg/ml), without the addition of iron-binding proteins to the medium, are able to proliferate. Moreover, it must be noted that, except for EGF, no protein was present in the synthetic medium. The ionized form of iron seems also to be important in these conditions inasmuch as maximal growth rate was obtained with the ferrous sulfate solution. This could be because ferric solutions are easily converted into ferric hydroxides, which precipitate and then release iron with more difficulty. If these results emphasize the iron requirement for cells growing in vitro and underline the importance of the ionic form of iron in a defined cell culture medium, iron uptake experiments would be of great interest and contribute to a fuller understanding of these data.

From our results, it can be suggested that two mechanisms of iron transport probably exist in HT 29 cells grown in defined medium: a passive one in the presence of a high concentration of iron in the medium and an active one mediated by a unique or different receptor for iron carrier proteins that are working at a low concentration of iron. The possibility of specific lactoferrin receptors on HT 29 cells must be considered. It was shown previously that specific Tf receptors, present on the membranes of reticulocytes, are inaccessible to Lf (18), and it was demonstrated that alveolar macrophages bind Lf in a specific manner without competition with Tf (19). Preliminary results obtained with HT 29 cells and shown in Table 1 suggest a specific binding of [^{135}I]Lf, whereas no significant binding inhibition was produced with an excess of Tf.

In addition, because it has been reported that lactoferrin may play a role in iron transport in the intestine (20) it would be of great interest if we could determine whether our results are only restricted to human HT 20 cells or if other cell types behave similarly in the presence of Lf.

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