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# A 41 kDa TRANSFERRIN RELATED MOLECULE ACTS AS AN AUTOCRINE GROWTH FACTOR FOR HL-60 CELLS[1]

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HL-60 cells produce an autostimulatory growth factor. Since the stimulatory effect of HL-60 conditioned medium is only observed in the absence of exogenous transferrin we have assayed HL-60 cells for the production of transferrin and found that they produce polypeptides which react with transferrin antibodies. $^{35}$S-methionine labelling, immunoprecipitation and subsequent separation by SDS-gel electrophoresis reveals the presence of a major transferrin related 41 ± 2 kDa species released by HL-60 cells. Physiological levels of iron salts completely abolish the requirement of exogenous transferrin which indicates that the endogenous transferrin related polypeptides in the presence of exogenous inorganic iron salts are sufficient for the proliferation of HL-60 cells provided insulin or related growth factors are present. The addition of transferrin receptor antibodies inhibits the stimulatory action of the endogenous transferrin related activity. © 1991 Academic Press, Inc.

For survival and growth most eukaryotic cells in culture require a medium supplemented with serum. In HL-60 cells, a human promyelocytic leukemia cell line, serum can be replaced with chemically defined medium (e.g.IMDM) when these cells are grown in suspension culture. In such a medium the presence of transferrin and supraphysiological concentrations of insulin is mandatory for growth to occur [1,2]. Through the action of transferrin the cells are provi-

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**Abbreviations:** IGF, insulin like growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazoliumbromide; ATCC, American Type Culture Collection; PBS, Phosphate buffered Saline; PPO, 2,5-diphenyloxazole; IMDM, Iscove's modified Dulbecco Medium; ELISA, Enzyme Linked Immunoadsorbent Assay; EGF, epidermal growth factor.

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ded with iron which is an important constituent of enzymes such as ribonucleotide reductase [3]. HL-60 cells possess transferrin receptors which are a prerequisite for the growth stimulatory effect of transferrin because anti-transferrin receptor antibodies inhibit the growth of HL-60 cells [4]. Since supraphysiological concentrations of insulin are required, it was argued that its action is mediated through the IGF-I receptor and proven that IGF-I is the physiological extrinsic growth stimulus [5]. Although most leukemic cells populations are completely dependent upon extrinsic growth factors there are also leukemic cells which produce their own specific growth factors for autocrine stimulation [6].

## MATERIALS AND METHODS

**HL-60 cell culture.** The HL-60 cell line was obtained from the ATCC (Rockville, Vg.). The cells were cultivated in IMDM supplemented with 5% fetal calf serum (both from Gibco) or under serum free conditions [1,7] in IMDM supplemented with 0.5 µg/ml bovine insulin (Sigma) and 5 µg/ml human transferrin (Sigma). The iron content of this non-iron supplemented medium is 10.14 µg/L (as determined by atomic absorption spectroscopy). The cell line was incubated at 37°C and 6% $CO_2$ and split twice a week. Every three months the cultures were evaluated for mycoplasma contamination.

**Gel chromatography of conditioned medium.** Medium of HL-60 cells conditioned in the absence of exogenous transferrin and insulin was concentrated 50x by speedvac evaporation and dialyzed against PBS containing calcium and magnesium ions (0.1 g/l) for 8 h and then centrifuged at 6500 x g. 2 ml of the concentrate were loaded on a Sephadex G75 column (Pharmacia). The column was then eluted with PBS at a flow rate of 14 ml/h. 3 ml fractions were collected, filter sterilized and 100 µl aliquots assayed for biological activity and immunoreactivity.

**Quantitative growth assay.** $0.125 \times 10^5$ cells/100 µl were incubated in IMDM in the presence of insulin (0.5 µg/ml) and 100 µl aliquots of the G75 column fractions eluate in 0.32 $cm^2$ wells. After 5 days the cell number was quantified by the MTT-assay [8]. In other experiments where iron salts [$Fe(NO_3)_3$] interfered with this assay cells were counted in a hemocytometer under the microscope.

**Solid phase assay for transferrin.** Rabbit polyclonal transferrin antiserum was obtained from Sigma. The ELISA was performed as described in [9].

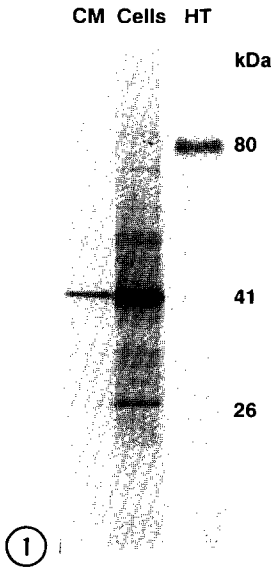
**Labelling of HL-60 polypeptides with $^{35}$S-methionine and immunoprecipitation with a polyclonal transferrin antiserum.** $1 \times 10^6$ cells per 1 ml batch were incubated with 50 µCi $^{35}$S-methionine (Amersham, specific activity 1410 Curies/mmol) for 3 days. The conditioned medium was freed of cells through centrifugation at 600 x g and transferred into a Centricon (Amersham) microconcentrator (cutoff 10 kDa). The immune precipitations were exactly carried out as described [10]. For comparative purposes human transferrin (Sigma) was electrophoresed on a parallel lane. After electrophoresis proteins were fixed in ethanol/acetate, the gels were soaked in a PPO-solution (Sigma) and dried. Autoradiography was carried out for 7 days.

**Incubation of HL-60 cells in the presence of transferrinreceptor-blocking antibody.** Partially purified transferrin related polypeptides (G75 fractions 10 and 11) were incubated with $1 \times 10^4$ HL-60 cells in 100 µl IMDM containing insulin in the presence of 50 µl of the monoclonal transferrin receptor anti-

body 42/6 (100 μg/ml)[11] commercially available from Dianova (Hamburg). As a control a monoclonal human EGF antibody produced in our laboratory [12] was used. After 3 days growth stimulation was quantified by the MTT-assay [8].

## RESULTS

When medium conditioned by HL-60 cells in the absence of insulin and transferrin was tested upon HL-60 cells a growth stimulatory effect was only observed when transferrin was also omitted from the indicator assay system (data not shown). Insulin, however, had to be present in the medium. When conditioned medium was concentrated and separated by gel chromatography the peak of growth promoting activity was observed in fractions 10 and 11 corresponding to an apparent molecular weight of 50 kDa (data not shown). Assay of the individual fractions for transferrin immunoreactivity revealed the presence of such an activity in these growth promoting fractions. To ascertain that the immunoreactive transferrin identified in the conditioned medium is indeed produced by the cells and not taken up while still incubated in the presence of transferrin (in earlier passages) HL-60 cells were assayed for the endogenous production of this polypeptide: after incubation in the presence of $^{35}S$-methionine cell associated as well as cell released transferrin was immunoprecipitated with transferrin antiserum and then separated on SDS-gels: medium conditioned by HL-60 cells revealed one major 41 ± 2 kDa species whereas cell extracts contained several bands in addition to this 41 kDa molecular species (Fig.1). The ratio of transferrin-bound radioactivity associated with the leukemic cells was 21:1 as compared to the one released into the medium. The endogenous production of a transferrin related molecule would implicate that HL-60 cells should grow in the absence of exogenous transferrin. However, growth was observed only when exogenous transferrin was present in concentrations of 0.05 μg/ml and higher in IMDM containing insulin (Fig.2). Since binding and uptake of iron is supposed to be the major role of transferrin (usually as iron-transferrin) additional iron was added to the medium of HL-60 cells in suspension culture. $Fe(NO_3)_3$ concentrations exceeding 1 μg/ml completely abolished the requirement of exogenous transferrin indicating that the endogenous sources were sufficient to bind and uptake the iron provided enough iron was present in the culture medium (Fig.3). 10 μg/ml ferric nitrate had the same effect as 5 or 50 μg/ml exogenous transferrin on the growth of the HL-60 cells as determined by day 5. When partially purified transferrin related activity (Fractions 10 and 11 of the G75 column) was incubated in the presence of the transferrin receptor antibody 42/6 (which specifically blocks the proliferative effect of transferrin [11]) with HL-60 cells its growth stimulating effect was reduced to 12% over the control (Fig.4). Monoclonal EGF antibody, however, had no effect on the growth stimulation caused by transferrin related activity.



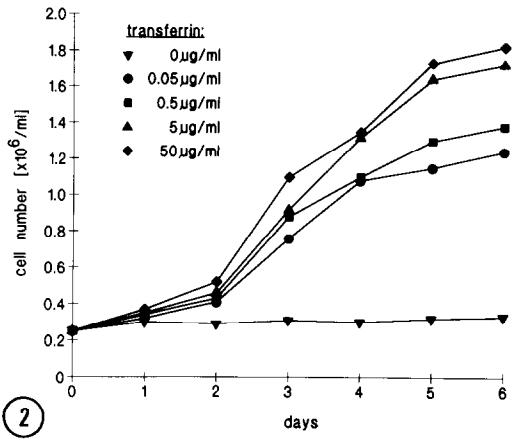
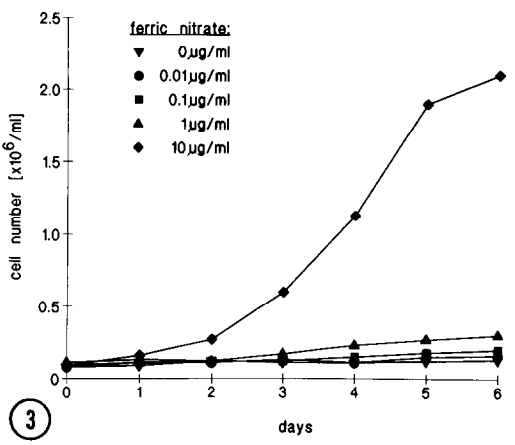




Fig.1. SDS-gel electrophoresis and autoradiography of $^{35}$S-methionine labelled proteins secreted by HL-60 cells (CM) and associated with HL-60 cells (cells). 5 µg 80 kDa human transferrin (HT) as a control.

Fig.2. Growth of HL-60 cells in the presence of various transferrin concentrations.

## DISCUSSION

HL-60 cells grow in the absence of exogenous transferrin provided enough iron can be taken up by the cells via endogenous iron binding molecules synthesized and secreted by the leukemic cells. We have identified a major 41



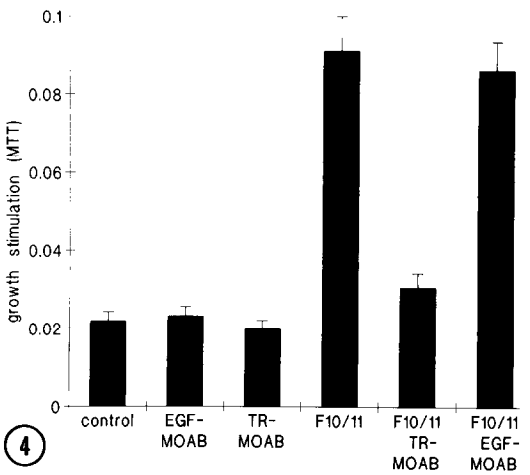




Fig.3. Growth of HL-60 cells in the absence of exogenous transferrin and after the addition of various amounts of $Fe(NO_3)_3$.

Fig.4. Growth of HL-60 cells in the presence of monoclonal antibodies directed against EGF (EGF-MOAB) or transferrin receptor (TR-MOAB), in the presence of fractions 10 and 11 of the G75 column chromatography and EGF-MOAB or TR-MOAB (n= 3 ± standard deviations).

kDa transferrin related molecule as the responsible agent. This molecule is also associated with the cells but in addition several minor high and low molecular weight immunoprecipitable substances can be recognized after a 72 hour labelling period. The molecular mass of 41 kDa is half of the 80 kDa transferrin normally produced by hepatocytes. Since the amino- and carboxy-halves of transferrin show an internal homology it has been proposed the protein propably evolved from a smaller ancestor by intragenic duplication thereby gaining an additional iron binding site. The 41 kDa species could be derived from a 80 kDa species produced by HL-60 cells and subsequently converted under the influence of intra- or extracellular proteases. However, we could not detect any 80 kDa species associated with HL-60 cells; moreover, in such a situation the 41 kDa species should also be produced from exogenous 80 kDa transferrin upon incubation with HL-60 cells which is not the case (data not shown). The observation that high molecular transferrin species are associated with the cells may suggest that leukemic cells produce a molecule similar to the transferrin related p97 which occurs in membranes of fetal and melanoma cells: such a molecule could serve as as a precursor to the 41 kD molecule since digestion of p97 with trypsin or papain produces a 40 kDa fragment [13]. p97 belongs to the family of iron binding proteins and crossreacts because of its structural similarity with transferrin antibodies. Another molecule which is recognized by transferrin antisera and therefore could account as origin for the transferrin related polypeptide reported here is 80 kDa lactotransferrin; however, it is not yet expressed in HL-60 cells [14]. Several tumor cell lines produce polypeptides that react with transferrin antibodies [e.g. 15]. The 41 kDa transferrin related polypeptide produced by HL-60 cells acts as an autocrine promotor of cellular proliferation since a transferrin receptor blocking antibody (used in a higher concentration than by [4]) inhibits its mitogenic effect. Prerequisite for the action of the 41 kDa molecule are, however, inorganic iron concentrations similar to the one in human plasma (which is about 2 µg/ml). The observation that physiological iron levels abrogate the presence of exogenous 80 kDa transferrin indicates that the growth stimulating effect is mediated through an increased iron uptake into the cell. This is supported by studies showing the antiproliferative action of iron chelators in HL-60 cells [16]. Our observations are in partial contrast to the statement of Collins [2] that requirement for insulin and transferrin is absolute as proliferation ceases if either of these compounds is removed from the serum free culture medium. Under appropriate iron supply the endogenous 41 kDa transferrin related molecule can fulfil the action of exogenous 80 kDa transferrin but only in the presence of insulin. This indicates that the actions of transferrin related and insulin related molecules may be interconnected as seen in other systems where the cycling

of transferrin receptors between the plasma membrane and endosomal membranes is regulated by growth factors such as IGF I [17].

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