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α-INTERFERON INDUCES DEPLETION OF INTRACELLULAR IRON CONTENT AND UPREGULATION OF FUNCTIONAL TRANSFERRIN RECEPTORS ON HUMAN EPIDERMOID CANCER KB CELLS

Michele Caraglia, Anna Maria Libroia, Stefania Corradino, Vincenzo Coppola, Rosario Guarrasi, Carmen Barile, Geppino Genua, Angelo Raffaele Bianco and Pierosandro Tagliaferri[1]

Cattedra di Oncologia Medica, Dipartimento di Endocrinologia ed Oncologia Molecolare e Clinica, Università "Federico II" Napoli, Italia

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SUMMARY: We have demonstrated that interferon-α (IFNα) upregulates the epidermal growth factor receptor (EGF-R) on human epidermoid carcinoma cells. Here we report that IFNα induces growth inhibition and upregulation of transferrin receptor (TRF-R) on epidermoid cancer KB cells. IFNα does not alter TRF-R affinity for its ligand and induces a two-fold increase of TRF binding sites. IFNα does not modify receptor internalization and cycling. Intracellular iron levels are known to regulate TRF-R expression: we have, therefore, evaluated whether changes in the iron content could be determined by IFNα. Iron levels are transiently increased after addition of fresh growth medium in untreated controls but not in KB cells exposed for 48 h to IFNα. Iron depletion is however completely reversed 24 h later when maximal TRF-R upregulation occurs in IFNα-treated cells. We suggest that IFNα-induced iron depletion elicits a homeostatic cellular response through upregulation of TRF-R.

Alpha-interferon (IFNα) induces growth inhibition of human tumor cells (1). This effect is mediated by specific receptors which are expressed at the tumor cell surface (2). Modulation of cellular response to peptide growth factors or changes in the receptor expression have been implicated in the mechanism of the antiproliferative effect of this cytokine (3,4).

We have described that IFNα inhibits the growth and upregulates the expression of the receptor for epidermal growth factor (EGF-R) in human epidermoid carcinoma KB cells (5). We have speculated that EGF-R modulation could be part of a homeostatic response of the tumor cells to the antiproliferative stimuli of the cytokine. In fact, we and others have observed that increased expression of the EGF-R is also induced by anticancer drugs at cytostatic concentration (6-9). Our working hypothesis has been recently reinforced by the finding of a cross-talk between IFNα and EGF (10).

[1]To whom correspondence and reprint request should be sent at Cattedra di Oncologia Medica, Università "Federico II", Facoltà di Medicina, Via S. Pansini 5, 80131, Naples, Italy. FAX:39 81 7462066.

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We have in fact demonstrated that human epidermoid carcinoma KB cells which have been exposed to IFNα at cytostatic concentration and show EGF-R upregulation become more sensitive to the effects induced by EGF on cell proliferation and tyrosine phosphorylation (10).

In order to evaluate whether other peptide growth factor receptors could be also modulated by IFNα we have investigated its effect on the cellular receptor for transferrin (TRF-R), an important regulator of cell proliferation (11). It functions by allowing intracellular iron uptake which is strictly required for cell growth (11,12). After receptor binding, iron saturated transferrin is internalized and endosomes are acidified, allowing transferrin and iron displacement from TRF-R (13). Subsequently, iron is accepted on intracellular storages of ferritin and both transferrin and unoccupied receptors recycle to cell membrane (13,14). TRF-R differs therefore from other peptide growth factor receptors in that it does not primarily activate intracellular signalling by membrane coupling intermediaries. Moreover, TRF-R expression is regulated by intracellular iron content at a transcriptional and post-transcriptional level (15,16).

In the present study we have found that TRF binding is increased by IFNα on human KB cells. We have therefore evaluated if TRF-R upregulation could be determined by an increased number of receptor proteins which could be recognised at the tumor cell surface by anti-TRF-R monoclonal antibody (MAb) which binds an epitope different from the TRF binding site (17). We have moreover studied the effects of IFNα on the process of TRF-R internalization and cycling. We have finally investigated the possible correlation between TRF-R modulation by IFNα and changes in intracellular iron storages.

MATERIALS AND METHODS

Cell culture. The human oropharyngeal epidermoid carcinoma KB cell line, obtained from the American Type Tissue Culture Collection, Rockville, MD, was grown in DMEM supplemented with heat inactivated 10% FBS, 20mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

[^{125}I]-TRF-binding. KB cells were seeded in 24-multiwell plates at 3 x 10^4/well. After 24 h and every 48 h thereafter, the medium was removed and fresh medium containing different concentrations of IFNα was added. After o/n incubation in serum-free medium (i.e. DMEM additioned with non essential aminoacids and vitamins) in the presence or absence of IFNα, the cells were washed twice with ice-cold PBS, additioned with 1 mg/ml of BSA and incubated for 4 h at 4°C with 200 μl/well of binding buffer (DMEM, HEPES 25mM, BSA 1mg/ml) containing increasing concentrations of [^{125}I]-TRF (200μCi/μg). Cells were washed four times with PBS/BSA and lysed in 0.5 ml/well of 20 mM HEPES, 1% Triton X-100, 10% glycerol. Cell-associated radioactivity was counted in a Beckman gamma-counter. The non-specific binding, determined by the addition of a 100-fold excess of unlabeled TRF and then subtracted for each point, never exceeded 5%. The value of TRF-R binding affinity and the receptor number were determined by Scatchard analysis of the [^{125}I]TRF-binding data (18), using EBDA/LIGAND, a computer program for fitting multiple binding site data (19).

Study of TRF internalization and cycling. KB cells were seeded in 24-multiwell plates at 2 x 10^4/well. KB cells were treated for 72 h with IFNα as described above. After o/n incubation in serum-free medium in the presence or absence of IFNα, the cells were washed twice with ice-cold PBS, additioned with 1 mg/ml of BSA. Then cells were incubated with [^{125}I]-TRF at

4 μg/ml for 30 min at 4°C and then at 37°C for different times. Cells were then washed 3 times at 4°C with PBS and incubated for 5 min at 4°C with 0.5 M NaCl-0.2 M acetic acid to remove surface bound TRF. The remaining internalized acid-resistant radioactivity in the cells was determined in a Beckman gamma-counter as previously described (20).

TRF-R live-cell radio-immuno assay (RIA). KB cells were harvested from subconfluent cultures and seeded in 96-well microtiter plates at 5 x 10^3/well. The cells were exposed for 72 h to 1,000 IU/ml of IFNα. After o/n incubation in serum-free medium in the presence or absence of IFNα, the growth medium was removed and 100 μl of 5% BSA (w/v) in DMEM and 0.08% (w/v) sodium azide were added to each well. After 60 min of incubation at 37 °C, the medium was removed and cells were washed with DMEM containing 5% BSA and 0.08% sodium azide. 50 μl of the appropriately diluted anti-TRF-R OKT9 were then added to each well. After incubation for 3 h at 4 °C the MAbs were removed and the cells were washed twice with PBS/BSA 5% (w/v). Then 75,000 cpm in 50 μl of [^{125}I]-labeled sheep anti-mouse IgG were added to each well for 60 min at 37 °C. Following incubation, cells were washed 3 times with PBS/BSA 5% (w/v) and 50 μl of 2 N NaOH were added to each well, adsorbed with a cotton swab and the radioactivity counted in a Beckman model gamma-counter. The background from the well that received only buffered DMEM was approximately 100 to 250 cpm, and was subtracted from that of the wells exposed to MAb OKT9 (6).

Determination of intracellular iron content. Subconfluent KB cells were grown in 100-mm dishes. The cells were exposed for different times to 1,000 IU/ml of IFNα. For cell extract preparation, the cells were washed twice with PBS, detached with gentle trypsinization, and counted with the haemocytometer. KB cells were washed once with PBS and then incubated for 30 min at 4 °C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1mM EDTA, pH 7.5, 10 mM Na(PO_4), pH 7.4,). The lysates were incubated in HCl 0.3 N for 20 min at 22 °C and then TCA-precipitated and centrifuged at 1,200 g for 15 min. The surnatants were mixed with chromogen (bathophenantroline, disulfonate 0.17 mM, sodium acetate 1.3 M, pH 4.6, sodium pyrosulfite 30 mM, p-(N-methyl)aminophenol 1.83 mM) and the absorbance was spectrophotometrically determined at the wavelenght of 546 nm. The iron concentration was calculated interpolating the absorbance of each sample in the preset iron standard curve and expressed as ng/10^6 cells.

RESULTS

IFNα determines a time- and dose-dependent increase of [^{125}I]TRF-binding in KB cells. We have previously reported that the addition of IFNα at concentrations of 100, 500 and 1,000 IU/ml induces growth inhibition of KB cells without cytotoxicity, as assessed by the trypan blue assay (5). IFNα, at these concentrations, also determines increased binding of [^{125}I]TRF on the same cells. Maximal TRF-R upregulation occurs after 72 h of exposure to each of the IFNα concentrations to whom KB cells have been exposed (Fig. 1A). At later times [^{125}I]TRF binding approaches the basal level. Interestingly, we have found that at the same experimental conditions the maximal EGF-R upregulation occurs after 48 h of IFNα exposure (10).

Exposure of KB cells to IFNα determines an increase in the number of [^{125}I]TRF binding sites without changes in the receptor affinity and induces enhanced cell labelling with anti-TRF-R MAb OKT9. We have evaluated if IFNα could modify the affinity of TRF-R. Scatchard analysis of [^{125}I]TRF binding data demonstrates that IFNα does not change TRF-R affinity (Fig 1B). One class of TRF-R is detectable with a K_d of about 4.8 nM either in

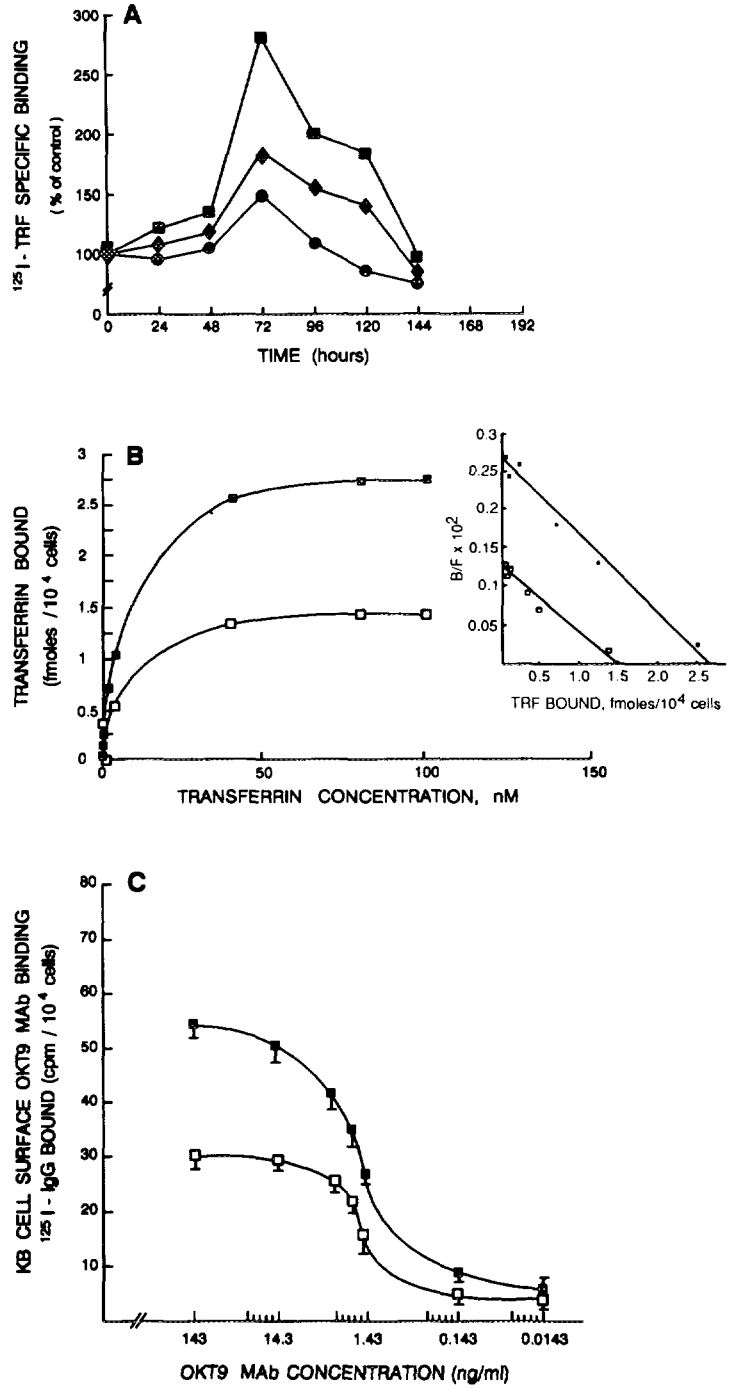

Figure 1. (A) Effects of different concentrations of IFNα on [^{125}I]TRF-binding: time-dependence. After medium withdrawal, fresh medium without IFNα or additioned with 100 IU/ml (●), 500 IU/ml (◆) and 1,000 IU/ml (■) IFNα was added every 48 h. [^{125}I]TRF binding was expressed as a percentage of control cultures. [^{125}I]TRF specific binding was determined in the presence of 100 nM unlabeled TRF. SE never exceeded 10%. Each point is the average of triplicate determinations.

Table 1. IFNα effects on TRF-R number and binding affinity

Treatment	Receptor number/cell	K_d/nM
none	$9x10^4$	4.3
72 h 1,000 IU/ml IFNα	$1.8x10^5$	4.8

unexposed and 72 h 1,000 IU/ml IFNα exposed KB cells (Tab. 1). The number of TRF binding sites is however doubled by IFNα treatment (90,000 and 180,000 in controls and IFNα exposed KB cells respectively) (Tab. 1). We have investigated if such effect could be determined by receptor unmasking or by a real increase of TRF-R molecules at the cell membrane. Accordingly, we performed a live cell RIA analysis of TRF-R expression at the KB cell surface by the use of MAbs which recognise the external portion of TRF-R at an epitope different from the TRF binding site (17). We have indeed found an increased labelling with OKT9 MAb of 1,000 IU/ml IFNα exposed KB cells and this effect quantitatively parallels the TRF-R upregulation as recorded with [^{125}I]TRF binding assay (Fig. 1C).

IFNα does not modify TRF-R internalization and cycling kinetics on KB cells. We have evaluated if the IFNα treatment of KB cells could modify TRF-R function. Since TRF-R internalization is a peculiar property of functional TRF-Rs we have studied the cycling kinetics of TRF-R with a [^{125}I]TRF binding assay (see Materials and Methods) (13). 72 h treatment with 1,000 IU/ml IFNα does not alter the internalization and cycling kinetics of TRF-R on KB cells, suggesting that upregulated TRF-Rs still preserve their physiological activity (iron internalization) (Fig. 2).

IFNα determines iron depletion on KB cells. Spectrophotometric assay demonstrates that intracellular iron levels are dependent from culture medium exchange: they are higher after addition of fresh medium and lower when the cells are left in exhausted medium (Fig. 3). When the cells are exposed to 1,000 IU/ml IFNα for 48 h, addition of fresh medium fails to increase intracellular iron levels, which however turned to be higher than controls 48 h later (Fig. 3). Intracellular iron content regulates TRF-R expression in normal and tumor cells (15,16). Interestingly, the lowering of intracellular iron is reversed when maximal TRF-R upregulation is recorded on KB cells.

(B) Binding curve and Scatchard analysis of TRF binding data on untreated controls and IFNα-treated KB cells. (A) [^{125}I]TRF binding to untreated (□) or 72 h 1,000 IU/ml IFNα-treated (■) KB cells. [^{125}I]TRF binding has been performed in the presence of different concentrations of [^{125}I]TRF. Points, averages from triplicate determinations. The *inset* shows Scatchard plots of specific binding. Data analysis was carried out with a curve fitting (multiple site) Scatchard analysis computer program.
(C) TRF-R-live cell RIA. Binding of anti-TRF-R MAb OKT9 to untreated (□) and 72 h 1,000 IU/ml IFNα-treated (■) KB cells as assessed by live-cell RIA which has been performed through the use of [^{125}I]sheep anti-mouse IgG and expressed as cpm/10^4 cells. Cells were drug-exposed as in [^{125}I]TRF binding experiments. Points, average of triplicate determinations. Bars, SD.

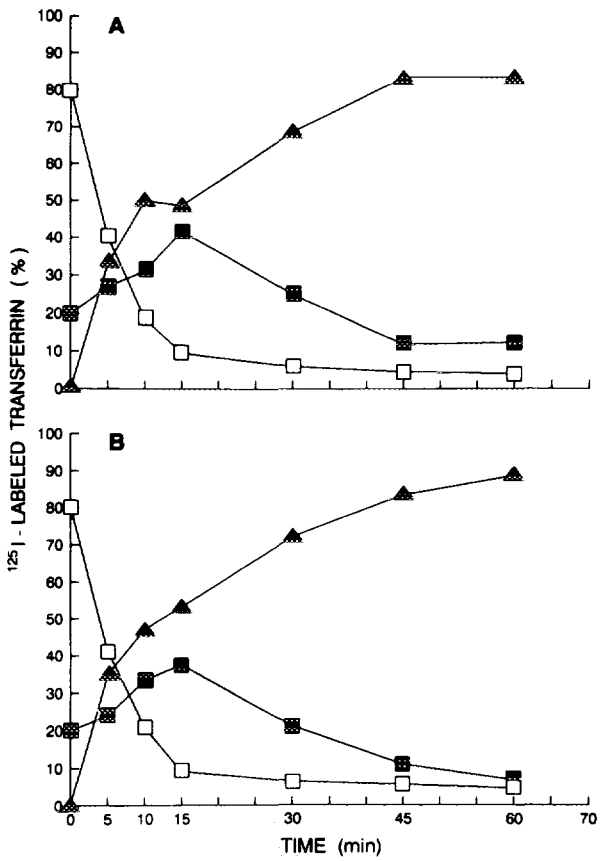

Figure 2. IFNα effects on TRF-R cycling of KB cells.Untreated (A) and 72 h 1,000 IU/ml IFNα–treated (B) KB cells were incubated with 4 μg/ml [^{125}I]TRF for 30 min at 4°C and then at 37°C for different times. Then medium-associated radioactivity (▲) and acid-sensitive radioactivity (□) ([^{125}I]TRF associated to cell surface and not internalized) were evaluated in a gamma-counter. The acid-resistant radioactivity (internalized [^{125}I]TRF)(■) in the cells was also determined. Points, average of triplicate determinations. SE never exceeded 5%.

DISCUSSION

The mechanisms of the antitumor activity of IFNα are still unknown even if immuno-mediated and direct antiproliferative effects have been postulated and often demonstrated in *in vivo* and *in vitro* systems (21,22). It has been proposed that direct growth inhibition of tumor cells induced by IFNα could be mediated by modulation of peptide growth factor receptor expression or function (3,4). Another mechanism of anticancer effect of IFNα has been related to metabolic changes in the target cells as the decrease of essential metabolites such as polyamines and modification of 2-deoxyglucose uptake (23). In this report we have demonstrated that iron depletion is induced by the treatment of human epidermoidal cancer KB cells with cytostatic concentrations of IFNα for 48 h. This effect however, is transient and intracellular iron levels are even increased at later times, when upregulation of functional

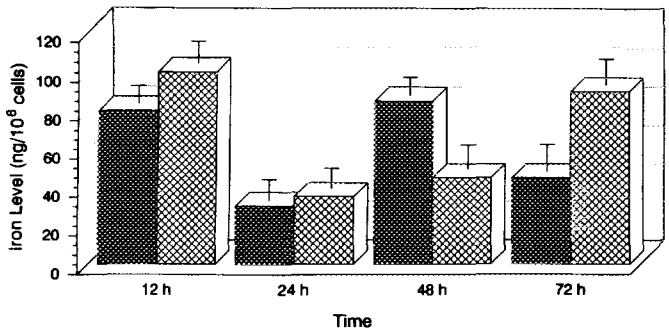

Figure 3. IFNα effects on the intracellular iron content of KB cells. Subconfluent KB cells were grown in 100-mm dishes. The cells were exposed for different times to 1,000 IU/ml of IFNα and then intracellular iron content was spectrophotometrically determined as described in Materials and Methods. (■) Unexposed KB cells, (▩) KB cells exposed to 1,000 IU/ml IFNα. Bars, SD.

TRF-R occurs in IFNα treated cells. In fact, high intracellular iron levels and upregulated expression of TRF-R are recorded 72 h after the beginning of KB cell exposure to 1,000 IU/ml IFNα. If we consider that expression of TRF-R is regulated by intracellular iron content at transcriptional and post-transcriptional level, a reasonable explanation of these events is that the receptor upregulation could take place as a homeostatic response to the depletion of an essential metabolite determined by IFNα exposure (15,16). We have recently reported that IFNα is able to induce increased expression of EGF-R also and enhanced sensitivity of KB cells to the growth promoting effects of EGF, which is paralleled by augmented tyrosine phosphorylation of cellular proteins and of the EGF-R itself (5). Taken together, these data suggest that an adaptive response could allow proliferation and survival of IFNα-inhibited epithelial cells through the upregulation of functional receptors involved in growth control. The study of the molecular events which occur in tumor cells exposed to cytokines with antiproliferative potential could disclose the understanding of behavioural changes of human tumor cells in stress conditions and at the same time allow a more rational use of such promising anti-cancer agents.

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