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ORIGINAL ARTICLE

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Interferon-alpha (Intron A) upregulates urokinase-type plasminogen activator receptor gene expression

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Abstract The regulation of urokinase plasminogen activator receptor (uPAR) gene expression by interferon-alpha (IFN-α, or Intron A) and interferon-gamma (IFN-γ) was studied in a HCT116 colon cancer cell line. uPAR mRNA levels were increased in a dose- and time-dependent manner in cells stimulated with IFN-α or IFN-γ. uPAR protein levels reflected IFN-α and IFN-γ induction of uPAR mRNA production. Cycloheximide, a protein synthesis inhibitor, also induced uPAR mRNA accumulation either alone or in combination with IFN-α or IFN-γ, suggesting that the effect on uPAR mRNA levels activated by IFN-α or IFN-γ does not require de novo protein synthesis. Both sodium butyrate and amiloride inhibited the uPAR mRNA levels induced by IFN-α or IFN-γ. These results may provide useful information for the treatment of patients receiving IFN-α or IFN-γ.

Keywords Colon cancer cell · Gene expression · Interferon (IFN) · Urokinase-type plasminogen activator receptor (uPAR)

Introduction

The involvement of the urokinase-type plasminogen activator (uPA) system in tumour invasion and metastasis has been suggested from a variety of model systems [2, 4, 32]. It is generally accepted that the uPA specifically binds to its receptor and provides inducible, transient and localised cell surface proteolytic activity [22]. The uPA receptor (uPAR) has been identified on the surface of many cell lines of both normal and neoplastic origin, including those of the colon. The binding of uPA to its receptor on the cell surface strongly enhances plasmin generation [10]. Receptor-bound uPA can be efficiently inhibited by two specific and fast-acting plasminogen activator inhibitors, PAI-1 and PAI-2 [11], and the receptor provides a mechanism for the internalisation of PAI-1 and PAI-2 inactivated uPA [13]. Moreover, the concomitant expression of uPA and uPAR has been shown to correlate strongly with a more invasive tumour cell phenotype. The regulation of uPAR gene expression, therefore, may be an important factor in localising and modulating cell surface plasminogen activation, the prerequisite for tumour invasion and metastasis.

There is evidence that uPAR protein production is regulated by certain growth factors and cytokines. The uPAR protein is inducible by phorbol myristate acetate (PMA) [21], interferon-gamma (IFN-γ) and tumour necrosis factor (TNF) in human monocytes [15]. Epidermal growth factor (EGF) was found to decrease the uPA binding capacity of a well-differentiated human colon carcinoma cell line, GEO, as a result of the reduced number of uPAR [5]. EGF caused a dose-dependent increase in the amount of radioactive uPA bound in a serum-free adapted CBScf colon cancer cell line [6]. Transforming growth factor beta-1 increased uPAR biosynthesis, mRNA levels and gene transcription in human A549 lung carcinoma cells [18]. Both TNF and IFN-γ induced uPAR mRNA and protein production in U937 cells, and IFN-γ but not TNF increased uPA binding capacity in these cells [26]. IFN-α and INF-γ potentiated all-*trans*-retinoic acid in its effects on uPA and plasmin activity, and on uPAR levels; however, IFN-α or IFN-γ alone did not have any potent effect on plasminogen activation [20].

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We have previously reported that TNF, PMA and cycloheximide (CHX) increased uPAR mRNA levels, and the first two also increased uPAR protein production in a highly invasive colon cancer cell line, HCT116 [8, 29]. Butyrate and amiloride, however, inhibited uPAR mRNA expression in the presence or absence of TNF and PMA. TNF acted at least partly at the transcriptional level and PMA in part at the post-transcriptional level [8, 29]. We have cloned and characterised human uPAR genomic DNA, which spans 23 kb and contains seven exons [30]. Sequence analysis revealed that the 5'-flanking region of the gene contains several putative *cis*-regulatory elements, including two AP-1 sites and a novel NF–kappa-B (κB)-like motif. Most recently, we have shown that a strong promoter is located in an 188-bp element between –141 and +47 relative to the transcription start site. An 81-bp region from –141 to –61, which contains a proximal AP-1 site and two Sp-1 motifs, is required for optimal uPAR promoter activity [9]. We have localised two AP-1 motifs in 70 and 183 bp, as well as a novel NF–κB site 45 bp upstream of the transcription start site [9, 17, 32]. The two AP-1 sites are required for both the constitutive and PMA-inducible expression of the uPAR gene in colon cancer cell lines [17]. These findings suggest that uPAR gene expression is controlled by multiple elements.

Recombinant IFN-α (Intron A), derived from *E. coli*, is used clinically in the treatment of chronic hepatitis B and C infection in immunocompetent patients, and in that of various cancers [3]. It exhibits many biological and biochemical effects on a number of cells and in animal models. Most significantly, it plays an important role in host defence against viral infections, and in resistance to experimentally-induced tumours [3]. Despite extensive studies on the interactions between IFN-α and other molecules, the regulation of uPAR mRNA expression by IFN-α has not been reported in detail. Our recent interest in the regulation of uPAR gene expression, and the role of uPAR gene expression in cancer development and metastasis, led us to examine the effects of IFN-α on uPAR mRNA expression in colon cancer cells. Both IFN-α and IFN-γ were found to be potent positive regulators of uPAR expression. Sodium butyrate and amiloride, on the other hand, inhibited the uPAR mRNA levels induced by IFN-α or IFN-γ.

Materials and methods

Materials

Human IFN-α (Intron A; 3×10^6 IU/ml), produced by the Pharmacy Department of Canberra Hospital (Canberra, Australia), was a gift from P. Pavli (Gastroenterology Unit, Canberra Hospital). Human IFN-γ (150 IU/ml) was donated by J. Medveczky (Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra). PMA, CHX, amiloride, butyrate, proteinase K, ribonucleic acid, and transfer RNA (tRNA) were obtained from Sigma (St. Louis, Mo.). [α-^{32}P]d-CTP and [α-^{32}P]d-UTP were from Amersham (U.K.). The 1.144-kb human uPAR cDNA [23] used in the present study was a gift from E.K.O. Kruithof (University Hospital, Geneva, Switzerland). Human 18S ribosomal DNA (18S rDNA) was provided by B.E.H. Maden (University of Liverpool, U.K.) [19]. T7 Quick Primer kit was purchased from Stratagene (U.S.A.). RQ1 deoxyribonuclease (RQ1 DNase) was purchased from Promega (Madison, Wis.). Ribonuclease A (RNase A) and Nick Columns were obtained from Pharmacia Biotech (Sweden). Nonidet P-40 was purchased from Boehringer Mannheim GmbH (Germany).

Cell culture

The human colon cancer cell line HCT116 has been described previously [7], and was obtained from the American Type Tissue Collection (ATCC). All cells were found to be free of *Mycoplasma* by Hoechst stain no. 33258. Cells were maintained in RPMI-1640 media supplemented with 10% fetal calf serum (FCS), L-glutamine, gentamycin and penicillin. Cells were incubated at 37°C in a 5% CO_2 incubator, washed with phosphate-buffered saline (PBS) and incubated overnight in RPMI serum-free media before stimulation. Immediately before harvest, cell viability was constantly found to be >90%.

cDNA probes

The α-^{32}P-labelled cDNA probes were prepared by the random priming method [1] using the T7 Quick Primer kit according to the manufacturer's instructions (Pharmacia Biotech, Sweden).

RNA preparation and northern blot analysis

Total cellular RNA was purified from cells using the guanidinium isothiocyanate method. The RNA samples were run on 1% formaldehyde-containing agarose gels, transferred onto a nitrocellulose membrane and further processed according to the method mentioned earlier [19]. Prehybridisation was carried out at 42°C for 16 h in 50% (vol/vol) formamide/5×SSC/5× Denhardt's solution/0.05 M Na_2PO_4, pH 6.7/salmon sperm DNA. Hybridisation with ^{32}P-labelled full-length uPAR cDNA was carried out in the same solution for 48 h at 42°C. The membrane was washed in 2×SSC/0.1% sodium dodecyl sulfate (SDS) once for 15 min at 42°C, and then in 0.1×SSC/0.1% SDS twice for 15 min at 55°C. The membrane was then exposed to Kodak XAR film at –70°C using intensifying screens, and multiple film exposure times were used to ensure linearity of band intensities. The intensities of mRNA bands in the autoradiographs were scanned and quantitated by a video densitometer (Model 620; BioRad). mRNA intensities were calculated relative to the intensity of the 18S rRNA internal control. The RNA molecular markers used were purchased from Promega.

Enzyme-linked immunosorbent assay for uPAR protein

The amount of uPAR protein from control and IFN-α- or IFN-γ-treated HCT116 cells was quantitated with a sandwich enzyme-linked immunosorbent assay (ELISA) by using a total uPAR ELISA kit (Imubind; American Diagnostica Inc.). 1×10^6 cells were plated in each well of six-well plates in 1.5 ml RPMI complete medium overnight for cells to become attached, and further cultured for 24 h in serum-free medium. After rinsing the cell monolayer three times with PBS, 0.5 ml of lysis buffer (0.01 M Tris–HCl, pH 8.1, 0.5% Triton X-100) was added and the cells were left at room temperature for 20 min. The cell lysates were then harvested and centrifuged at 8,160 *g* for 5 min at 4°C to obtain the post-nuclear fraction. The aliquots of supernatant were stored at –70°C for testing by ELISA. The ELISA test was carried out according to the manufacturer's instructions. One hundred microlitres of total uPAR standard and diluted samples were added to the precoated micro-test wells in a moist chamber and incubated overnight at 4°C. All measurements were performed in duplicate. The wells were washed four times with wash buffer (0.1% Triton-100 in PBS). One

hundred microlitres of detection antibody (biotinylated anti-human uPAR) was then added to each well and incubated for 1 h at room temperature. After four washes with wash buffer, diluted enzyme conjugate [streptavidin–horseradish peroxidase (HPR)] was added to each well and incubated for 1 h at room temperature. The wells were washed four times with wash buffer, 100 µl of substrate solution (3,3',5,5'-tetramethylbenzidine; TMB) was added and the plate was incubated for 20 min at room temperature until a blue colour developed. The enzymatic reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 to each well. By then, the solution had turned yellow and the absorbances were read within 30 min on a micro-test plate reader at a wavelength of 450. Absorbances (optical density; o.d.) were calibrated to standards in each experiment using purified human total uPAR over the range 0–10 ng/ml, due to daily variation in the o.d. produced by uPAR standards.

Results

Time- and dose-dependent induction of uPAR mRNA accumulation by IFN-α and IFN-γ

To study the effects of IFN-α and IFN-γ on uPAR mRNA accumulation in HCT116 cells, total RNA was extracted from cells treated with IFN-α or IFN-γ and analysed by northern blotting. The levels of uPAR mRNA were very low in the unstimulated HCT116 cells, while the 1.4-kb uPAR mRNA levels were markedly increased in the IFN-α- or IFN-γ-treated cells (Figs. 1 and 2). The northern blots were stripped and rehybridised with a human 18S rDNA probe to enable the assessment of the relative amount of mRNA in each lane. As shown in Fig. 1A, after IFN-α stimulation, uPAR mRNA levels were increased at 0.5 h, then increased eight-fold at 8 h and maintained at this high level for 48 h. Following IFN-γ stimulation, uPAR mRNA levels were also increased at 0.5 h, reached a maximum 10-fold increase at 24 h and then remained constant for up to 48 h (Fig. 1B). The uPAR mRNA levels increased in a dose-dependent manner at IFN-α concentrations of 10^2–3×10^5 IU/ml (Fig. 2A) and at IFN-γ concentrations of 5–1,000 IU/ml (Fig. 2B). No significant difference was observed in 18S rRNA, which served as an internal control.

Effect of CHX on uPAR mRNA accumulation in HCT 116 cells induced by IFN-α or IFN-γ

To test whether the induction of uPAR gene expression by IFN-α or IFN-γ was dependent on de novo protein synthesis, CHX was used to inhibit protein synthesis. Cells were pre-incubated with CHX (20 µg/ml) for 30 min to ensure inhibition of protein synthesis at an early stage, and then IFN-α or IFN-γ was added for a further 4 h. Northern blot analysis and scanning of the autoradiograms showed that CHX increased the amount of uPAR mRNA induced in cells treated with either IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml; Fig. 3). The results suggest that the effects of IFN-α and IFN-γ did

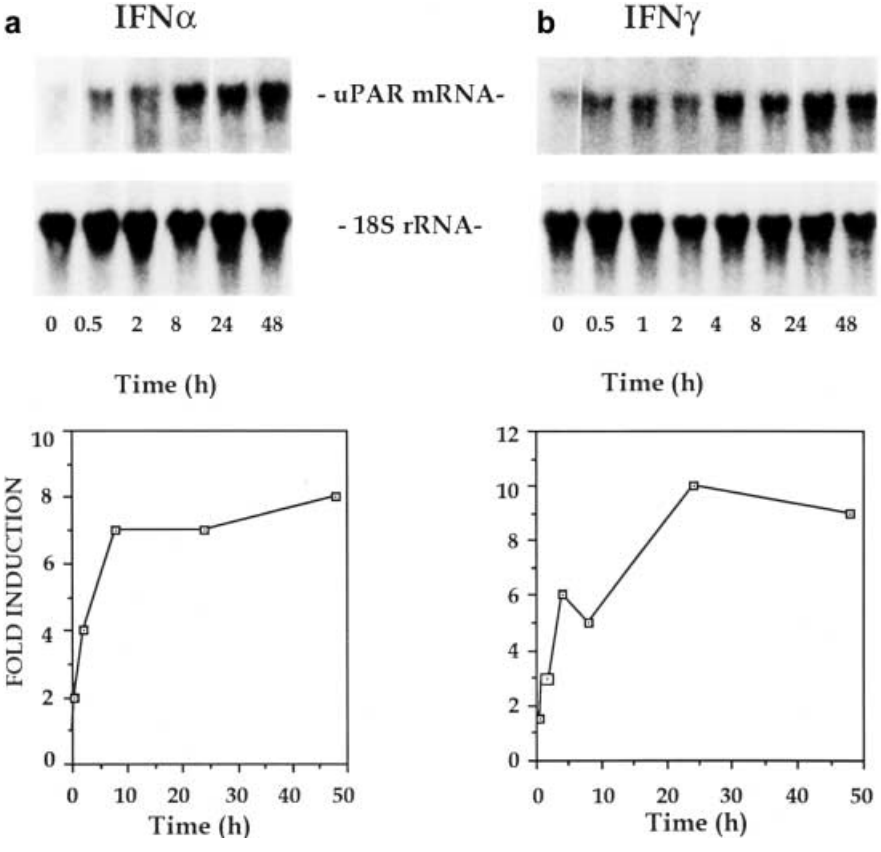

Fig. 1A, B. Time course of uPAR mRNA accumulation in IFN-α- or IFN-γ-stimulated HCT116 cells. Twenty micrograms of each RNA sample from cells treated with IFN-α (10^4 IU/ml; **A**) or IFN-γ (200 IU/ml; **B**) for the indicated time periods was used for northern blot analysis and hybridised sequentially with ^{32}P-labelled uPAR cDNA and 18S rDNA probes. Autoradiographic exposure time was 24 h (for uPAR as probe) and 2 h (for 18S rDNA as probe). The corresponding densitometry results are shown below. Each experiment was repeated at least three times, and representative data are shown in the figure

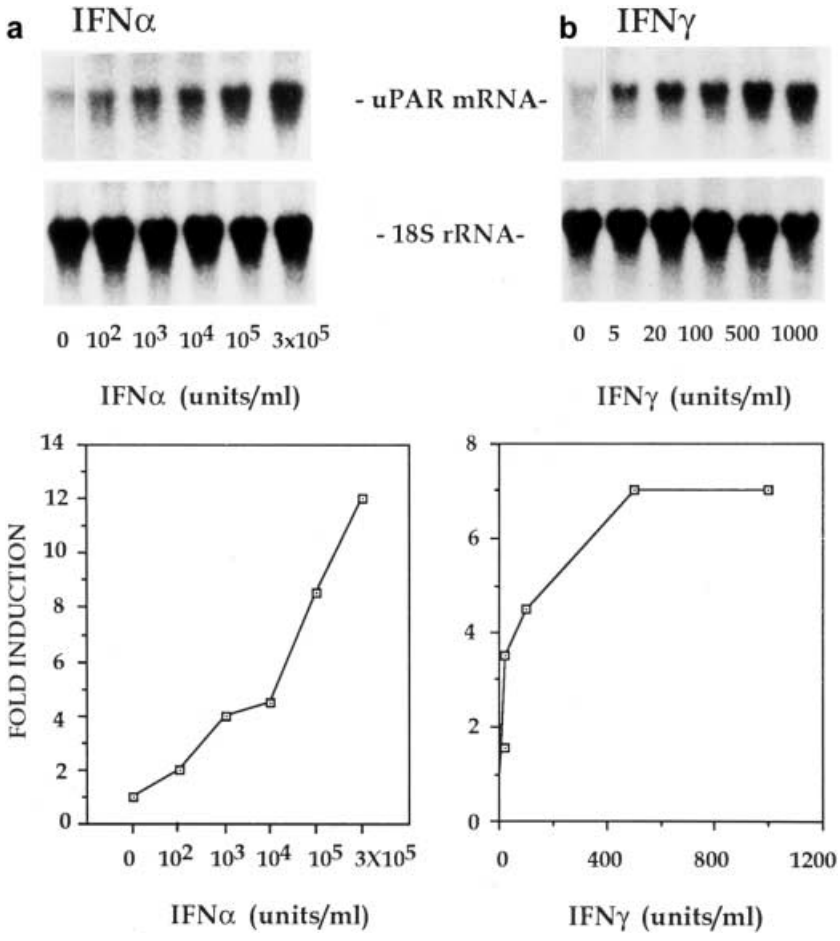

Fig. 2A, B. Dose-dependent induction of uPAR mRNA accumulation by IFN-α or IFN-γ in HCT116 cells. Twenty micrograms of each RNA sample from cells treated with different concentrations of IFN-α (**A**) or IFN-γ (**B**) for 4 h was used for northern blot analysis. The same northern blot was hybridised with ^{32}P-labelled uPAR cDNA and 18S rDNA probes, as indicated. Autoradiographic exposure time was 24 h (for uPAR as probe) and 2 h (for 18S rDNA as probe). The corresponding densitometry results are shown below. Each experiment was repeated at least three times and representative data are shown in the figure

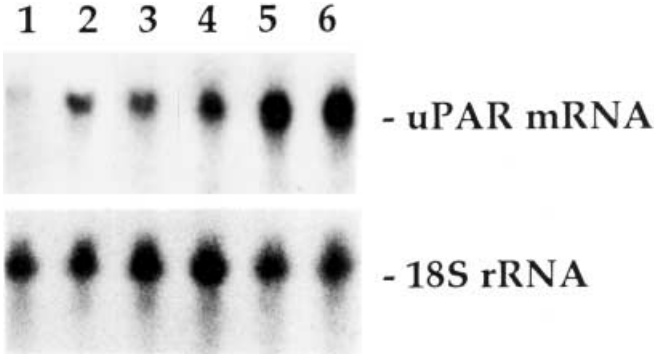

Fig. 3. Effect of CHX on the accumulation of uPAR mRNA induced by IFN-α or IFN-γ. Twenty micrograms of each RNA sample, from cells treated with CHX (20 μg/ml) alone or in combination with IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml) was used for northern blot analysis. The same northern blot was hybridised with ^{32}P-labelled uPAR cDNA and 18S rDNA probes, as indicated. Autoradiographic exposure time was 24 h (for uPAR as probe) and 2 h (for 18S rDNA as probe). *Lane 1*: HCT116; *lane 2*: IFN-α; *lane 3*: IFN-γ; *lane 4*: CHX; *lane 5*: CHX plus IFN-α; *lane 6*: CHX plus IFN-γ

not require de novo protein synthesis. No significant difference was observed in 18S rRNA, which served as an internal control.

Inhibition of uPAR mRNA expression by amiloride and sodium butyrate in HCT116 cells stimulated with IFN-α or IFN-γ

As amiloride and sodium butyrate inhibited uPAR mRNA expression in HCT116 cells [8, 29], the effects of these two reagents on the uPAR mRNA accumulation induced by IFN-α or IFN-γ were examined. As shown in Fig. 4, both of them inhibited the increased expression of uPAR mRNA in HCT116 cells stimulated with IFN-α or IFN-γ. Again, no significant difference was observed in 18S rRNA, which served as a control.

Effects of IFN-α and IFN-γ on uPAR protein levels

To determine whether the changes in uPAR mRNA reflected the alterations in uPAR protein levels in HCT116 cells induced by IFN-α and IFN-γ, an ELISA was carried out. After treatment with IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml) for 24 h, cell lysates were prepared with lysis buffer (0.01 M Tris–HCl, pH 8.1, 0.5% Triton X-100) and ELISA was performed as described in Materials and methods. As shown in Fig. 5, the level of uPAR protein in unstimulated HCT116 cells was

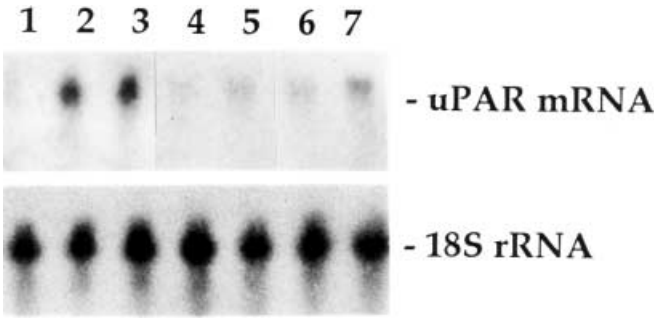

Fig. 4. Inhibition of uPAR mRNA expression by amiloride or sodium butyrate in HCT116 cells stimulated by IFN-α or IFN-γ. Twenty micrograms of each RNA sample, from cells treated with different reagents (IFN-α: 10^4 IU/ml; IFN-γ: 200 IU/ml; sodium butyrate: 2.5 mM; amiloride: 1.5 mM) was used for northern blot analysis. The same northern blot was hybridised with ^{32}P-labelled uPAR cDNA and 18S rDNA, as indicated. Autoradiographic exposure time was 24 h (for uPAR as probe) and 2 h (for 18S rDNA as probe). *Lane 1*: HCT116; *lane 2*: IFN-α; *lane 3*: IFN-γ; *lane 4*: IFN-α plus butyrate; *lane 5*: IFN-γ plus butyrate; *lane 6*: IFN-α plus amiloride; *lane 7*: IFN-γ plus amiloride

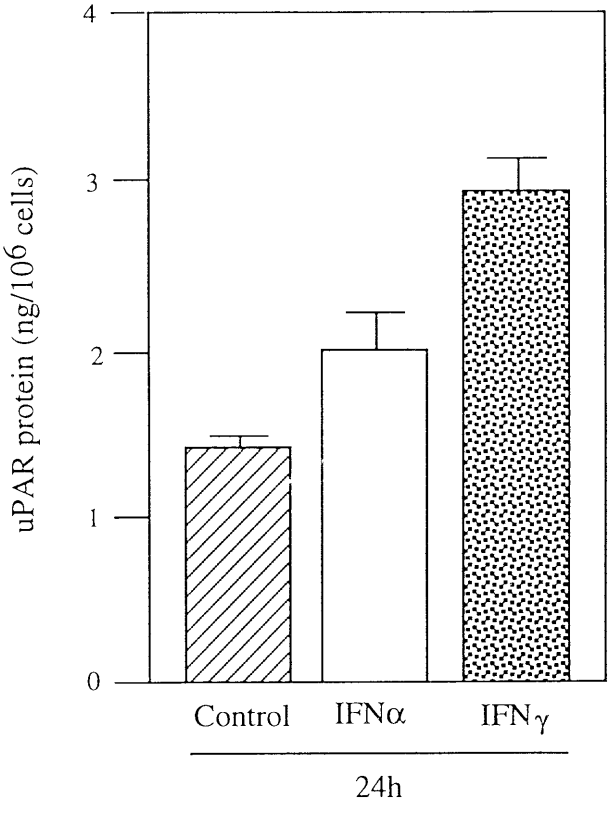

Fig. 5. Effects of IFN-α and IFN-γ on uPAR protein production. Cell lysates from control HCT116 cells, HCT116 cells treated with IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml) for 24 h were prepared and assayed for uPAR protein levels by ELISA. Experiments were performed in duplicate and data expressed as mean ± SD. The assays were repeated three times

1.39 ng/10^6 cells. After IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml) stimulation for 24 h, uPAR protein levels increased to 2.0 ng/10^6 cells (1.4-fold) and 2.92 ng/10^6 cells (2.1-fold) respectively. uPAR protein levels, therefore, reflected IFN-α and IFN-γ induction of uPAR mRNA levels in HCT116 cells.

Discussion

In this paper, a colon cancer cell line, HCT116, was chosen as a model system for studying the regulation of uPAR mRNA expression by IFN-α and IFN-γ. The uPAR mRNA accumulation in HCT116 cells was positively regulated by IFN-α and IFN-γ in a time- and dose-dependent manner, and this upregulation by IFN-α or IFN-γ was negatively regulated by sodium butyrate and amiloride. These increases in uPAR mRNA levels were parallel to the increases in cellular uPAR protein production. Moreover, CHX increased uPAR mRNA expression either alone or in combination with IFN-α or IFN-γ.

IFN-α (Intron A) has been used in the clinical setting as an effective treatment for some diseases including chronic hepatitis B/C infection and various cancers [3]. The data presented in this paper show that both IFN-α and IFN-γ have a significant effect on uPAR mRNA and protein levels. These results suggest that IFN-α and IFN-γ may play a role via uPAR in several biological processes including angiogenesis, monocyte migration, cancer metastasis, trophoblast implantation and wound healing. Our data may assist in the examination of side effects during the process of IFN-α or IFN-γ treatment for cancer patients and also for patients with other diseases.

To determine whether the induction of uPAR mRNA by IFN-α or IFN-γ was dependent on de novo protein synthesis, CHX was used to inhibit protein synthesis. As shown in Fig. 3, CHX increased the amount of uPAR mRNA induced by either IFN-α (10^4 IU/ml) or IFN-γ (200 IU/ml). The results suggest that the effects of IFN-α or IFN-γ did not require de novo protein synthesis. CHX blocks the peptidyl transferase reaction on ribosomes, and thus inhibits protein synthesis. This protein synthesis inhibitor is also a potent and fast-acting inducer of uPAR mRNA in HCT116 cells [8, 29]. An additive effect is seen when IFN-α or IFN-γ and CHX are used in combination, as in this study. It suggests that protein synthesis is not required for the effects of IFN-α or IFN-γ, and also indicates that CHX either blocks the synthesis of a negatively-regulating protein or of an enzyme that degrades uPAR mRNA. Other studies have suggested that uPAR mRNA contains one AUUUA sequence in the 3'-untranslated region [23], common to those mRNAs that are known to be subject to fast and selective degradation as reported for granulocyte–macrophage colony-stimulating factor mRNA [25].

This paper also shows that amiloride or sodium butyrate can block uPAR mRNA in HCT116 cells induced by IFN-α or IFN-γ. Amiloride is a specific inhibitor of sodium channels in many cellular transport systems. It is also a competitive inhibitor of uPA [28] and its receptor

[29], but not of tissue plasminogen activator (tPA), plasmin, plasma kallikrein or thrombin, indicating that it does not have a general inhibitory action on cell metabolism. There is evidence that amiloride given to rats in their drinking water completely prevented pulmonary metastases when rat mammary adenocarcinoma cells were injected intravenously [29].

Another uPAR inhibitor discussed in this paper is sodium butyrate, which is a short-chain fatty acid produced in the lumen of the human colon by bacterial fermentation of dietary fibre [27]. At the concentrations present in the lumen it inhibits cell proliferation, stimulates cell differentiation [12] and induces apoptosis of colonic carcinoma cells in culture [12, 14, 27]. However, the effects of butyrate on the genes implicated in the cell surface proteases that may determine adenocarcinoma invasion and metastasis are poorly understood. We have previously reported that both amiloride and sodium butyrate inhibited the expression of uPAR mRNA in HCT116 cells [8, 29]. These observations, combined with the inhibitory effects of amiloride and butyrate on uPAR expression reported in this paper, encourage further research into the mechanisms of amiloride or butyrate-induced inhibition of metastasis that may offer fresh insight into the prevention of adenocarcinoma invasion and metastasis.

It has been shown that the cooperation of two cell types may play an important role for plasmin generation in both normal and tumour tissues [16]. The different locations of uPA, uPAR and PAI-1 producing cells in various types of cancer indicate that a complex interaction may take place between cancer cells and stromal cells, in which the cancer cells could recruit or induce stromal cells to produce the necessary molecules as critical balance between uPA and PAI-1 which are required for the development of invasive and/or metastatic tumours. The reciprocal interaction between the cancer cells and the stromal cells may be mediated by growth factors, hormones and cytokines, including IFNs. It is possible that the IFNs, either alone as shown in the present work or in combination with other regulatory molecules, induce the various activities necessary for plasmin generation under physiological conditions. It would be interesting and very important to clarify the role of individual components of the uPA system in the cancer metastasis process in order to better understand why this regulation apparently fails in cancer.

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