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Characterization of a Hepatic Proliferation Inhibitor (HPI): Effect of HPI on the Growth of Normal Liver Cells—Comparison With Transforming Growth Factor Beta

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Improvements in the purification of a hepatic proliferation inhibitor (HPI) from adult rat liver have yielded a product that has an inhibitory activity 1,000-fold greater than previously reported. The growth inhibitory activity, which could be eluted from SDS-PAGE at 17–19 kilodaltons (kD), was compared to that of transforming growth factor beta (TGF-β). The ID_{50} of the HPI preparation in Fischer rat liver epithelial cells was 50 pg/ml (2.5 pM) compared to a value of 260 pg/ml (10.4 pM) obtained for pure human TGF-β. Both inhibitors also modulated the stimulation of DNA synthesis in primary hepatocytes by either epidermal growth factor or a growth stimulatory activity prepared from serum of hepatectomized rats. The ID_{50}s of HPI and TGF-β in these cells were 250 pg/ml and 40 pg/ml, respectively. In contrast to TGF-β the growth inhibitory activity of HPI was unaltered in the presence of an antibody raised against TGF-β. The possible mechanism of action of HPI is discussed.

Key words: growth inhibition, primary hepatocytes, liver epithelial cells

The characterization of factors that control cellular proliferation is central to the understanding of both normal and neoplastic growth. Although much progress has been made in the last few years toward the identification and characterization of polypeptide growth factors that stimulate cell proliferation, there has been much less success in the isolation and characterization of growth inhibitory polypeptides with the notable exception of transforming growth factor beta (TGF-β).

The purification of a hepatic proliferation inhibitory protein (HPI) was reported in 1982 by McMahon et al [1]. This protein, which was isolated from normal rat liver, specifically and reversibly inhibited cell division and DNA synthesis in a number of nonmalignant rat liver epithelial cell types, although it had no effect on transformed liver cells [2]. Subsequent analysis has indicated that the preparation was

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not homogeneous, the apparent single band on SDS-PAGE (26 kD) being due to a contaminant [3]. Recently, a number of improvements have been made to the purification scheme for this protein which have yielded a product that has an inhibitory activity 1,000-fold greater than that previously reported [3].

The best characterized endogenous inhibitor of normal cell proliferation is the multifunctional polypeptide TGF-β, a 25-kilodalton (kD) dimer consisting of two identical chains of 112 amino acids [4]. It has been demonstrated that TGF-β is a strong inhibitor of proliferation of many primary and secondary cell types including embryo fibroblasts [5], T and B lymphocytes [6,7], keratinocytes [8], and bronchial epithelial cells [9]. Recently, several other proteins including a growth inhibitor from the conditioned medium of monkey kidney cells (BSC-1) [10], a cartilage-inducing peptide (CIF-A) isolated from bovine bone [11], and a differentiation inhibitor (DI) secreted by Buffalo rat liver cells [12] have been shown to be similar to or identical with TGF-β. In addition, other regulatory peptides including inhibin [13] and Mullerian inhibitory substance (MIS) [14] have been found to have structural homology to TGF-β.

The wide tissue distribution of TGF-β [4], its molecular weight, and the finding that it is a potent inhibitor of DNA synthesis in primary hepatocytes [15–18] has prompted speculation that TGF-β and HPI are identical molecules [15]. The recent observation that TGF-β is produced by oval cells within the liver (personal communication, N. Fausto) has added further support to this possibility. In the present study we have further characterized HPI by means of SDS-PAGE and have utilized freshly isolated hepatocytes and a normal diploid rat liver cell line to compare the growth inhibitory activities of TGF-β and HPI.

MATERIALS AND METHODS

Cells and Materials

The cell line of normal liver epithelial cells (RLE) was maintained in culture as described previously [16] and for this study was used between the 13th and 18th passage in vitro. Primary hepatocytes were isolated from a male F344 rat (150 g) by the two-step collagenase perfusion technique of Evarts et al [19]. Waymouth MB 752/1 medium was purchased from Gibco Laboratories (Grand Island, NY); Hams F-12 medium was a product of Biofluids Inc. (Rockville, MD); and the cell culture supplements, collagen type IV, and [^{125}I]-epidermal growth factor (EGF) (122 μCi/μg) were obtained from Collaborative Research (Lexington, MA). "Defined" fetal bovine serum was from HyClone Laboratories Inc. (Alexandria, VA). Methyl-[^{3}H]-thymidine (102 μCi/μg) was purchased from Amersham Corporation (Arlington Heights, IL). Pure TGF-β, isolated from human platelets [20], and a rabbit antibody raised against human TGF-β [21], which is specific to mature 25 kD TGF-β (personal communication, A. Roberts), were gifts of Dr. Michael Sporn, NCI. HPI was purified by the method of Krutzsch et al [3]. The most highly purified form available was used for all studies except in the gel elution study where a partially purified preparation was used. All other materials were obtained from sources described previously [22].

Elution of HPI From SDS-PAGE

A crude preparation (1 μg total protein) containing HPI activity (ID_{50}: 500 pg/ml for RLE cells) was subjected to SDS-PAGE [23] under nonreducing conditions

using a 12.5% polyacrylamide gel. Gel slices (5 mm) were excised and extracted at 4°C for 16 hr in 1 ml Ham's F-12 medium containing 10% "defined" fetal bovine serum (FBS). The extracts were then dialyzed at 4°C for 48 hr against PBS (pH 7.4) prior to the analysis of 10-μl aliquots for growth-inhibitory activity using RLE cells.

Purification of Hepatocyte Growth Factor

A crude preparation of a serum-derived hepatocyte growth factor (serum-HGF) was prepared by heparin affinity chromatography of serum obtained from F-334 rats (180 g) 24 hr after partial hepatectomy as described previously [24]. The active fraction, tested for stimulation of primary hepatocyte proliferation in serum-free cell culture, was further purified by gel filtration (Sephadex G-100), concentrated, and assayed for protein content by the method of Bradford [25] using bovine serum albumin as the standard.

Measurement of DNA Synthesis in Primary Hepatocytes

Freshly isolated hepatocytes were washed with Ham's F-12 medium containing 10% FBS supplemented with insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium (6.25 ng/ml), and dexamethasone (20 ng/ml). The cells were seeded in 96-well microtiter plates, precoated with type IV collagen, at a density of 4.5×10^4 cells/cm^2 and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. The medium was replaced at 4 hr with serum-free defined medium. This consisted of Waymouth's medium supplemented with insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium (6.25 ng/ml), dexamethasone (20 ng/ml), bovine serum albumin (1.25 mg/ml), and linoleic acid (5.35 μg/ml). Following incubation for 16 hr, the medium was replaced with serum-free defined medium containing EGF (10 ng/ml) or HGF (50 μg/ml) together with HPI or TGF-β at various concentrations. After 4 hr methyl-[^{3}H]-thymidine (0.5 μCi) was added to each well and the cells were incubated for a further 48 hr prior to measurement of DNA content and DNA synthesis as described by Richards et al [22].

Measurement of RLE Cell Proliferation

RLE cells were seeded into 96-well microtiter plates at a density of 2.7×10^4 cells/cm^2. The effect of HPI and TGF-β on the extent of DNA synthesis and cell proliferation was assayed by the modification of the method of Richards et al [22] as described by McMahon et al [16].

Effect of Anti-TGF-β Antibody on Activity of Inhibitors

The effect of a rabbit antibody raised against human TGF-β on the activity of HPI and TGF-β was examined using RLE cells. Samples containing the antibody (8.3 μg/ml) and various concentrations of HPI or TGF-β in Ham's F-12 media containing 10% FBS were incubated at 37°C for 4 hr prior to their analysis for inhibitory activity. The IgG fraction (8.3 μg/ml) of normal rabbit preimmune serum [21] was used as a control.

Measurement of EGF Binding

Radioreceptor binding assays were performed using A-431 human carcinoma cells according to previously described procedures [26]. The effect of HPI pretreatment of cells on EGF-receptor binding was studied by incubating the A-431 cells for

2 hr at 37°C in a humidified atmosphere of 5% CO_2/95% air with 280 pg/ml HPI prior to the analysis of [^{125}I]-EGF binding.

RESULTS

The elution of growth-inhibitory activity from a polyacrylamide gel following SDS-PAGE analysis of a partially purified sample previously shown to possess HPI activity (ID_{50}:500 pg/ml) indicated a molecular weight for this activity of 17–19 kD (Fig. 1). The analysis of gel extracts from a lane in which bovine serum albumin was electrophoresed in parallel with the HPI sample indicated that this inhibitory activity was specific to the HPI sample and not due to gel contaminants.

The growth-inhibitory activities of HPI and TGF-β were initially compared using freshly isolated primary hepatocytes. These cells were stimulated to undergo DNA synthesis by the addition of either EGF (10 ng/ml) or HGF (50 μg/ml). These concentrations produced about a tenfold increase in methyl-[^{3}H]-thymidine incorporation over basal levels, although there was no significant change in cell number over the 48-hr time course of the experiment. Both HPI and TGF-β produced a dose-dependent decrease in the stimulation of DNA synthesis by EGF, and at the highest concentrations tested, both inhibitors were able to block completely the stimulation of DNA synthesis in these cells (Fig. 2). Essentially identical results were obtained when DNA synthesis was stimulated with HGF. The ID_{50} concentrations for the HPI- and TGF-β-dependent inhibition of DNA synthesis, 250 pg/ml and 40 pg/ml, respectively, were independent of the mitogen employed. It must be noted that the purity of

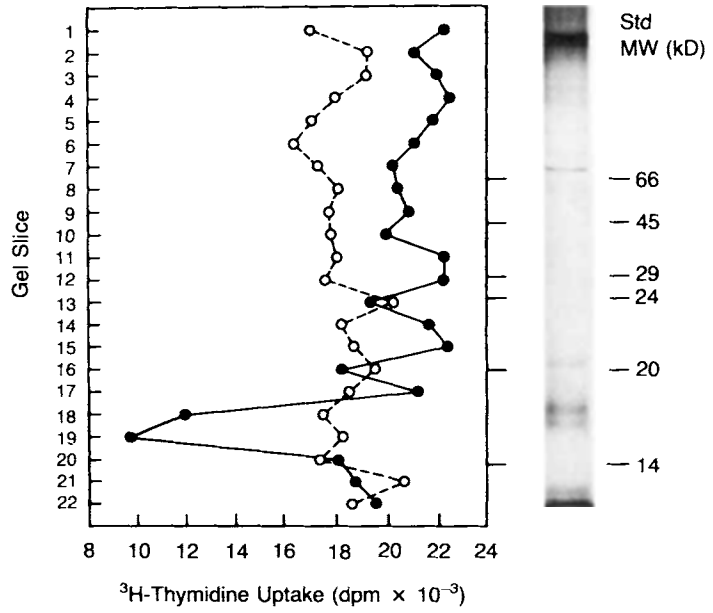

Fig. 1. Elution of HPI from SDS-PAGE. A crude preparation (1 μg total protein) containing HPI (ID_{50} = 500 pg/ml) was subjected to SDS-PAGE (12.5% acrylamide). Gel slices (5 mm) were extracted with Ham's F-12 medium containing 10% FBS. The extracts were then dialyzed against PBS (pH 7.4) prior to analysis for inhibitory growth activity using RLE cells (●). A control sample containing 1 μg albumin was analyzed in parallel (○). A silver-stained gel of the HPI preparation is also shown.

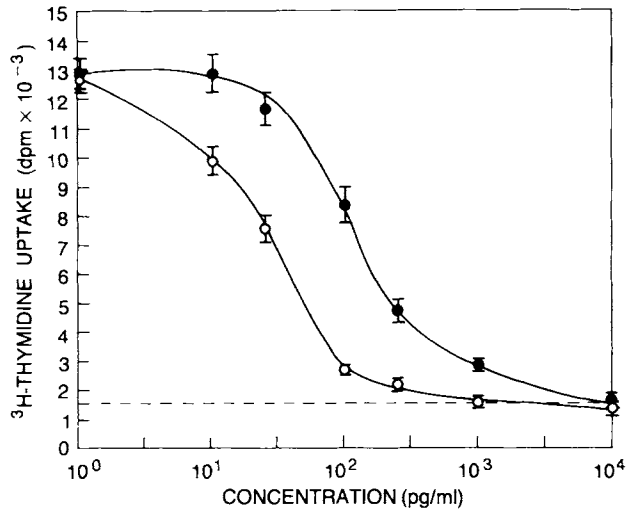

Fig. 2. The effect of HPI and TGF-β on EGF-induced DNA synthesis in primary hepatocyte cultures. EGF (10 ng/ml) together with various concentrations of HPI (●) or TGF-β (○) were added to primary hepatocyte cultures, and the incorporation of methyl-[^{3}H]-thymidine into cellular DNA was measured for 48 hr (mean ± SEM, n = 6). The dotted line indicates the basal level of DNA synthesis in the absence of EGF.

the HPI preparation is as yet unknown, and so the total protein concentration of the preparation has been used for the determination of the ID_{50} concentrations for HPI.

The effect of HPI and TGF-β on DNA synthesis in RLE cells was also examined using a 96-well microtiter assay. However, as these cells proliferate during the time course of the assay, the extent of DNA synthesis, measured as incorporation of methyl-[^{3}H]-thymidine, was normalized to the cell number by dividing the [^{3}H]-dpm value with the DNA fluorescence value. Previous studies have demonstrated that this fluorescence value has a linear relationship with cell number [22]. HPI and TGF-β inhibited both DNA synthesis and cell proliferation of RLE cells in a dose-dependent manner, and the ID_{50} concentrations of each were 50 pg/ml and 260 pg/ml, respectively (Fig. 3). It is of note that although the highest dose of TGF-β was almost completely able to block DNA synthesis in RLE cells, the maximal inhibition that could be obtained with HPI was about 75%.

In studies designed to distinguish the growth-inhibitory activities of HPI and TGF-β in RLE cells, it was found that the activity of HPI was essentially unchanged following its incubation with an anti-TGF-β antibody (Fig. 4A). In contrast, the dose-response curve for TGF-β inhibition of RLE cell DNA synthesis was shifted to the right following treatment of the TGF-β sample with the anti-TGF-β antibody (Fig. 4B). The ID_{50} for TGF-β was increased from about 300 pg/ml to 1.6 ng/ml in the presence of the antibody.

The possibility that the inhibitory effects of HPI are mediated by an effect on EGF-receptor binding was investigated using A431 cells as only a limited degree of [^{125}I]-EGF binding was obtained using RLE cells. Preincubation of A-431 cells with HPI (280 pg/ml) for 2 hr at 37°C (5% CO_2/95% air) had no effect on the subsequent binding of EGF to its receptor (Fig. 5).

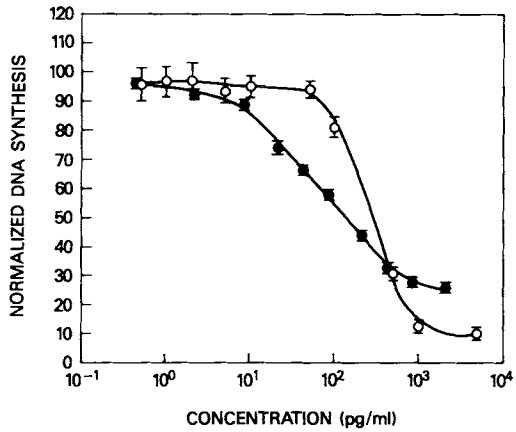

Fig. 3. The effect of HPI and TGF-β on the proliferation of RLE cells. Various concentrations of HPI (●) or TGF-β (○) were added to proliferating RLE cell cultures. The incorporation of methyl-[^{3}H]-thymidine incorporation, normalized by cell number was then measured in a 2-hr pulse following a 48-hr incubation (mean ± SEM, n = 8).

DISCUSSION

The finding that TGF-β is a negative growth regulator in addition to being able to stimulate the proliferation of some cell types [27,28] has lent further support to the hypothesis that cellular homeostasis is the result of a delicate balance between the influence of growth factors and growth inhibitors. Although the identification of polypeptide growth factors has proceeded at an ever-increasing rate over the past few years, only a very few growth-inhibitory proteins have been isolated, and many of these have been found to be related or identical with TGF-β. This has led to the speculation that the liver-derived hepatic proliferation inhibitor is identical with TGF-β. In this paper we provide evidence that TGF-β and HPI are distinct growth inhibitors, although both produce similar effects on the growth of liver-derived normal cells in vitro.

The previously determined molecular weight and isoelectric point for HPI [1], 26 kD and 4.65, respectively, were based on the analysis by SDS-PAGE and isoelectric focusing of a preparation which was subsequently shown to contain a major contaminant responsible for the single band that was observed on staining of the gels with Coomassie blue [3]. Analysis of the present HPI preparation using chromatofocusing indicated an isoelectric point of 5.5 and elution of the activity through a gel filtration column (ACA44) was consistent with a molecular weight of about 26 kD [3]. However, the elution of growth-inhibitory activity from SDS-PAGE (Fig. 1) indicates a molecular weight for HPI in the range 17–19 kD. This apparent discrepancy may be due to the fact that the electrophoresis was performed in the presence of SDS in which reversible protein denaturation occurs, while the gel filtration elution was carried out in conditions favoring the native conformation of proteins. In addition, molecular weight data obtained from gel filtration experiments may be unreliable owing to protein-protein or protein-gel interactions. The silver-stained protein bands which appeared to comigrate with the activity on SDS-PAGE (Fig. 1) were from contaminants in the relatively crude HPI preparation used and were found to have no inhibitory activity. The elution of growth inhibitory activity from SDS-PAGE pro-

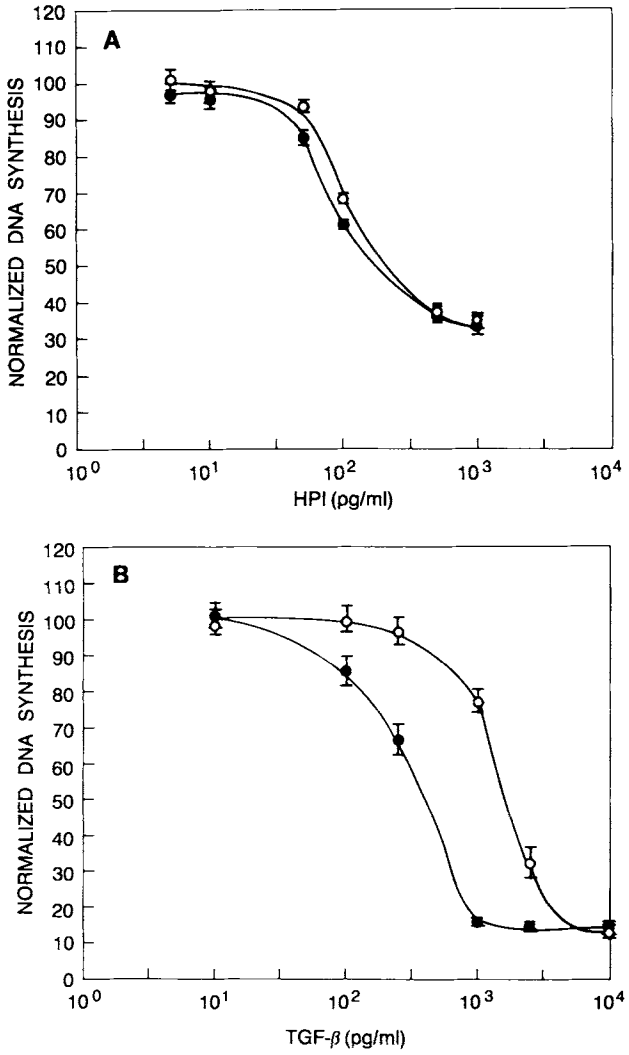

Fig. 4. The effect of anti-TGF-β antibody on the inhibition of RLE cell proliferation by TGF-β or HPI. **A:** Various concentrations of HPI were incubated for 4 hr with an anti-TGF-β antibody (8.3 μg/ml; ○) or an IgG control (●). The samples were then added to RLE cell cultures and normalized DNA synthesis was measured after 48 hr (mean ± SEM, n = 8). **B:** Various concentrations of TGF-β were incubated for 4 hr with an anti-TGF-β antibody (8.3 μg/ml; ○) or an IgG control (●). The samples were then added to RLE cell cultures, and DNA synthesis, normalized against cell number, was measured after 48 hr (mean ± SEM, n = 8).

vides further evidence that this activity is not due to TGF-β as previous experiments have demonstrated that TGF-β is eluted from SDS-PAGE at a position corresponding to a molecular weight of 25 kD [29]. Other physical/chemical studies performed on the highly purified HPI preparation also indicate that HPI and TGF-β are different polypeptides [3].

This is the first report demonstrating the inhibitory activity of HPI on growth-factor-stimulated DNA synthesis in primary hepatocyte cultures. The uptake of

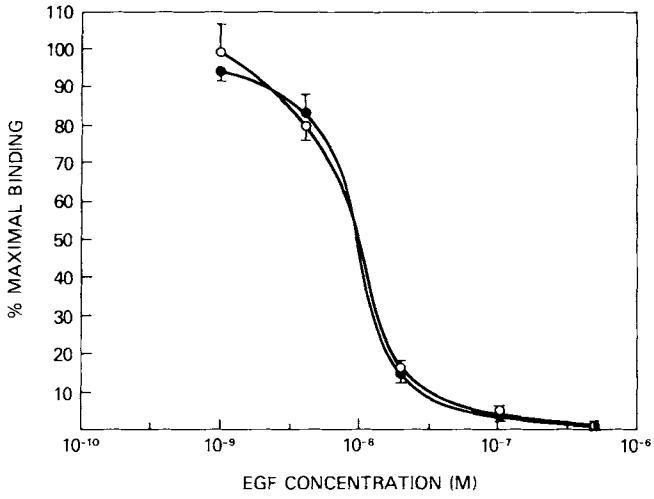

Fig. 5. The effect of preincubation with HPI on subsequent [^{125}I]-EGF binding to A431 cells. HPI (280 pg/ml) was incubated with A431 cells for 2 hr at 37°C. The cells were then washed and examined for EGF-receptor binding (●). A control experiment in which there was no preincubation of cells with HPI was performed in parallel (○) (mean ± SD, n = 4).

methyl-[^{3}H]-thymidine was studied in serum-free cell cultures plated at low cell density, conditions favoring maximal mitogen-induced DNA synthesis. The increase in methyl-[^{3}H]-thymidine uptake induced by either EGF or serum-HGF was totally abolished in the presence of the highest concentrations of HPI or TGF-β tested. Serum-HGF is a serum form of a growth-stimulatory activity for hepatocytes released by platelets which is different from platelet-derived growth factor (PDGF) and EGF [30]. Both HPI and TGF-β also inhibited DNA synthesis and cell proliferation in RLE cell cultures. However, in contrast to the results obtained with the primary hepatocyte cultures, the ID_{50} for the HPI preparation was lower than that for TGF-β. In addition, the dose-response profiles for the two inhibitors were different for the RLE cells, a much steeper curve being observed for TGF-β than HPI. Although TGF-β, at the highest concentrations tested, produced almost a complete inhibition of DNA synthesis and cell proliferation, the maximal inhibition that could be achieved using HPI was about 75%. Further studies have indicated that the RLE cells become more resistant to the growth-inhibitory effects of HPI with increasing passage number. These results not only indicate that TGF-β and HPI are different molecules but also suggest that their inhibitory activity in these cells is mediated through different mechanisms. This finding is supported by previous studies which have demonstrated that HPI is a reversible inhibitor of proliferation of the RLE cells [1], while the inhibition of RLE cell proliferation produced by TGF-β appears to be irreversible and may in fact lead to a more differentiated phenotype [16]. A similar finding has been reported when human bronchial epithelial cells were treated with TGF-β [9]. Additional evidence indicating that HPI and TGF-β are different polypeptides is provided by the results of the experiments utilizing an anti-TGF-β antibody. Although the antibody was able markedly to reduce the inhibitory effects of TGF-β on the proliferation of RLE cells, it had no effect on the inhibition of growth induced by

HPI. The antibody (8.3 μg/ml) was able to abolish completely the inhibitory effect of the normal ID_{50} concentration of TGF-β for these cells.

The mechanisms by which HPI and TGF-β mediate the growth arrest of normal cells are unknown. It has been clearly demonstrated that TGF-β does not compete with EGF for binding to the EGF-receptor [31,32], although it has been reported that TGF-β treatment produces a transient decrease in EGF-receptor affinity in NRK cells [33,34]. The binding of EGF to A431 cells was unaltered following pretreatment of these cells with HPI for 2 hr. A study on the effect of EGF binding to RLE cells was not possible as the level of specific binding of [^{125}I]-EGF was very low even in the absence of HPI. In addition it was not possible to examine the competition of HPI with [^{125}I]-EGF for specific binding to the EGF-receptor owing to the limited amounts of HPI available. Although these results suggest that HPI has no effect on EGF-receptor affinity, it is necessary to confirm this finding in cells known to be responsive to the growth-regulatory effects of HPI. The effect of HPI pretreatment on the binding of EGF to its receptor in hepatocytes is currently under investigation. Thus the present studies demonstrate that HPI is a growth inhibitor that is distinct from TGF-β. Work is currently in progress to determine the tissue specificity of HPI and its possible involvement in the neoplastic process.

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