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Involvement of Insulin-like Growth Factors-I and -II and their Receptors in Medroxyprogesterone Acetate-induced Growth of Mouse Mammary Adenocarcinomas

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The role of the insulin-like growth factors (IGFs) system was investigated in hormone-dependent (HD) and -independent (HI) *in vivo* lines of the medroxyprogesterone acetate (MPA)-induced mammary tumor model in Balb/c mice. IGF-II protein and message showed a three- to four-fold increase in HD lines growing in MPA-treated mice, as compared with HD tumors growing in untreated mice. Progression to a hormone-independent phenotype in all these lines was accompanied by a high constitutive expression of IGF-II. Similar IGF-I mRNA levels were detected in HD and HI lines. Both IGF-I and -II messages arose from the malignant epithelial cells, as shown by *in situ* hybridization studies. A significant decrease in Man-6P/type II IGF-R content was detected in HD tumors growing in MPA-treated mice as compared with HD lines growing in untreated mice. On the other hand, in HI tumors, notwithstanding high IGF-II synthesis, the levels of Man-6P/type II IGF-R remain high. Competitive inhibition and affinity labeling studies showed an almost exclusive binding of IGF-II to Man-6P/type II IGF-R on tumor membranes. The involvement of IGFs in the growth of epithelial primary cultures of the C4-HD line was evaluated. Exogenous IGF-I potentiated MPA stimulatory effect at concentrations of 50–100 ng/ml. Treatment of C4-HD cells with antisense oligodeoxynucleotides (ASODNs) to type I IGF-R and to IGF-II RNA resulted in a dose-dependent inhibition of MPA-mediated cell proliferation. The inhibition caused by IGF-II ASODNs could not be overcome by the addition of IGF-II up to 150 ng/ml. ASODNs to type I IGF-R at 40 μg/ml reduced by 75% the number of type I IGF-R; ASODNs to IGF-II at 1 μM decreased by 83% the levels of IGF-II protein. Our results provide support for the involvement of IGF-I and -II in MPA-induced mammary tumor growth by autocrine pathways. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

We had formerly studied the involvement of growth factors (GFs) modulating tumor growth in the medroxyprogesterone acetate (MPA)-induced mammary tumor model in BALB/c mice [1–5]. In this model, tumor lines which are maintained through subcutaneous syngeneic passages, usually retain the biological features of the parental tumor; they express progesterone (PR) and estrogen receptors (ER) and disclose a progestin-dependent (HD) growth behavior [1]. Through successive passages we obtained progestin-independent (HI) lines [1]. The

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availability of these HD and HI tumor lines and the fact that we were able to develop primary cultures [6], have allowed us to focus our interest on the role of GFs in certain aspects of carcinogenesis such as progestin-mediated growth changes, and transition from a HD to a HI state.

The insulin-like growth factor (IGFs) system is specially attractive in breast cancer since experimental evidence suggests its involvement as mediator of steroid hormone-stimulated growth in mammary tumor cells [7–9]. The IGFs system is composed of two ligands (IGF-I, IGF-II) that bind to three types of receptors: type I IGF receptor, mannose-6-phosphate (Man-6P)/type II IGF receptor, and an insulin/type I IGF hybrid receptor. IGFs also have high affinity for a family of specific binding proteins (IGF-BPs) that modulate their cellular effects (for review see [10]). It has been reported that estrogens induce or regulate the expression of type I IGF-R and IGF-II [11–13] in different experimental models and, conversely, that the latter can induce loss of estrogen dependence for growth [14, 15]. Substantially less information is available about the interactions between progestins and the IGFs system in mammary cancer. Both type I IGF-R and IGF-II are regulated by progestins in breast cancer cell lines [16] and the blockade of either IGF-I or type I IGF-R using antibodies resulted in the inhibition of progestin-stimulated growth of MCF-7 breast cancer cells [17] and carcinogen-induced rat mammary tumors [18]. An additional observation supporting the progestins/IGFs interactions is that the treatment of MCF-7 cells with IGF-I increases the levels of progesterone receptor (PR) protein [19].

In a former work, we found similar levels of IGF-I protein expression and of type I IGF-R [3] in HD lines and in their HI selected variants.

In the present study, we demonstrate that MPA-mediated tumor growth is associated with an up-regulation of IGF-II protein and mRNA levels in HD tumor lines. We also show that progression to a hormone-independent phenotype is associated with a high constitutive expression of IGF-II. We studied the cellular localization of IGFs and their receptors and performed competitive inhibition and affinity labeling studies in order to characterize IGFs binding parameters on tumor membranes. In addition, we used an antisense strategy to evaluate the involvement of type I IGF-R and IGF-II in the MPA-induced growth of epithelial cultures of a HD tumor line.

MATERIALS AND METHODS

Animals and tumors

The experiments were carried out in virgin female BALB/c mice. All animal studies were conducted in accordance with the highest standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals. Hormone dependent ductal tumors (C4-HD, D5-HD, and C7-HD), originated in mice treated with 40 mg MPA every 3 months for 1 year, are maintained by serial transplantation in MPA-treated animals [2]. Their hormone independent counterparts, C4-HI, D5-HI, and C7-HI developed from HD tumors growing in mice that were not treated with MPA, approximately a year after the inoculum [1].

Primary cultures and cell proliferation assays

Primary cultures of epithelial cells from C4-HD tumors, growing in MPA-treated mice, were performed as previously described [6]. Purity of the epithelial cultures was evaluated by cytokeratin staining [6]. Cells were incubated in DMEM/F12 (Dulbecco's modified Eagle's medium:Ham's F12, 1:1, without phenol red, 100 U/ml penicillin, and 100 μg/ml streptomycin), with 1% steroid-stripped fetal calf serum (ChFCS) in the presence or absence of MPA 10 nM and IGF-I or IGF-II at concentrations ranging from 5 to 100 ng/ml. After a 24 h incubation, 50% of media was replaced by fresh media and cells were incubated for another 24 h in the presence of 0.2 μCi of ^{3}H-thymidine (NEN, Dupont, Boston, MA; specific activity: 70–90 Ci/mmol). Cells were then trypsinized and harvested. Assays were performed in octuplicates; mean and standard deviation were calculated for each solution tested. The differences between control and experimental groups were analyzed by ANOVA followed by Tukey *t*-test between groups. In former experiments we demonstrated that thymidine uptake correlates with the number of cells/well [6].

Antisense studies

Type I IGF-R: antisense (5′ TCC TCC GGA GCC AGA CTT) and sense (5′ AAG TCT GGC TCC GGA GGA) oligodeoxynucleotides (ODNs) correspond to codons 21–26 of the signal sequence of the subunit of type I IGF-R preceding the proreceptor sequence. IGF-II: antisense (5′ CCC ATT GGT ACC TGA AGT TG) and sense (5′ CAA CTT CAG GTA CCA ATG GG) ODNs correspond to the mouse IGF-II mRNA translation initiation region. In addition, a scrambled sequence (5′ CGC CTT GTG ATA CGA CTT AG) with the same overall oligodeoxynucleotides content as the IGF-II antisense oligo was used. ODNs were purchased from CyberSyn (Lenni, PA) or from NucleiCo (Buenos Aires, Argentina).

The effect of ODNs was studied as follows: in a Falcon 96-well microtitreplate, 0.2 ml/well of a cell suspension were seeded at a concentration of 10^5 cell/ml. After attachment (24–48 h), the cells were incubated for 48 h with medium (serum used in antisense studies was heated at 65°C for 30 min to inactivate

exonucleases) containing the indicated concentrations of ODNs. The media were changed every 24 h adding fresh ODNs. After incubation, (^{3}H)-thymidine was added and incorporation was determined as described above.

Acid-extraction and IGF-II purification by C18 chromatography

Tumor samples (600–1000 mg) were homogenized 1:4 (w/v) in 1 M acetic acid and extracted and purified as described [20].

IGF-I and -II receptor binding assay

Recombinant human IGF-I and IGF-II, kindly provided by Dr James Merryweather (Chiron, Emeryville, CA), were iodinated and purified as described [20].

Binding to tumor membranes. Aliquots of 100 μl of HD and HI tumor microsomal membranes (30–50 μg protein), obtained as described by Barenton *et al.* [21] to avoid IGF-BP contamination, were used. IGF-I and IGF-II binding studies were carried out as described [20]. Assays were done in triplicate and results were analyzed by the Scatchard method [22].

Binding to intact cell monolayers. To prepare cultures for binding studies, C4-HD epithelial cells, obtained as described [6], were seeded in 6-multiwell plastic dishes and grown until monolayers reached 90% confluence (approximately 1×10^6 cells/well). Growth medium was aspirated and cells were washed three times with phosphate buffered saline (PBS). IGFs binding studies were performed using the same concentration of ligands described for tumor membranes in 1 ml of binding buffer (NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1.2 mM, $MgS0_4$ 1.2 mM, HEPES 50 mM, pH 7.5) containing 2 mg/ml of BSA for 2 h at 4°C. The incubation was terminated by aspirating the radioactive medium and by washing the monolayer three times with ice-cold PBS. The cells were then treated with 1% Triton X100-PBS buffer, the solution transferred to disposable tubes, and finally, radioactivity was counted. Assays were performed in triplicate and results were analyzed as above.

Cross-linking, electrophoresis and autoradiography

Aliquots from 200 to 500 μg of membrane protein from the tumors were affinity labeled using 2 nM ^{125}I-IGF-I or -II in the presence or the absence of an excess of 500 ng of nonradioactive IGF-I or -II as described [20]. Affinity cross-linkings were also performed on intact monolayer cultures of C4-HD epithelial cells. Bindings of ^{125}I-IGF-I or -II to cells were carried out as described above. Cells were then washed three times with ice-cold binding buffer and incubated for 15 min at 4°C with this buffer containing 0.2 nM disuccinimidyl suberate (DSS) (Pierce, Rockford, IL). The reaction was quenched with Tris 1 M, EDTA 1 mM, pH 7.4 and cells were scraped off in presence of Tris 10 mM, EDTA 1 mM, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin, 1 μg/ml leupeptin. The suspended cells were collected by centrifugation at 12,000*g*, and solubilization buffer (10 mM Tris, 1 mM EDTA, pH 7.4 with 1% Triton and protease inhibitors) was added to the pellets. After a 40 min incubation at 4°C, insoluble material was pelleted and sample buffer was added to the supernatant. Electrophoresis on 7.5% polyacrylamide gels was performed according to the method of Laemmli [23]. Molecular weight markers were: myosin (200,000 kDa), phosphorylase B (97,400 kDa), BSA (68,000 kDa), ovalbumin (43,000 kDa), chymotrypsinogen (25,700 kDa) (BRL, Gaithersburg, MD).

IGF-II radio-receptor assay (RRA)

The amount of IGF-II present in the HD and HI tumor extracts was quantified using the IGF-II radioreceptor assay described by Guerra *et al.* [20]. RRA experiments were repeated a minimum of three times with each individual tumor extract.

IGF-II Western blotting

Lysates were prepared from C4-HD cells growing in medium with 1% ChFCS + 10 nM MPA and from cells growing in medium with 1% ChFCS + 10 nM MPA + ODNs. After washing in ice-cold PBS, cells were lysed in buffer RIPA (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) with the following protease inhibitors: PMSF 0.5 μM, leupeptin 10 μg/ml, benzamidine 1 μM, pepstatin 5 μg/ml, aprotinin 5 μg/ml. Cell lysates were centrifuged at 14,000*g* for 20 min at 4°C and protein content in the supernatant was determined using a Bio-Rad kit (Richmond, CA). Proteins were solubilized in sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and subjected to SDS-PAGE on a 15% gel. Proteins were electroblotted on to nitrocellulose. Membrane was blocked with PBS, 0.1% Tween 20 (PBST) and immunoblotted with a 1:200 dilution of a IGF-II goat polyclonal antibody raised against a peptide corresponding to amino acids 159–178, mapping at the carboxy terminus of the mouse IGF-II precursor (M-20, Santa Cruz Biotechnology, Santa Cruz, CA). After washing six times for 5 min with PBST, the membrane was incubated with 1:1000 biotinylated anti-goat antibody (Amersham International, U.K.), washed as before and incubated with a 1:1000 streptavidin–peroxidase (Amersham). Enhanced chemiluminescence (ECL) was performed according to manufacturer's instructions (Amersham). The relative intensities of the IGF-II bands were quantitated using a LKB (Bromma, Sweden) Ultroscan XL densitometer.

Probes

IGF-I and -II mouse cDNA clones were kindly provided by Dr P. Rotwein (Washington University School of Medicine, St Louis, MO). The IGF-I clone, constructed in a pGEM 4 vector (Promega, Madison, WI), was linearized with BamH I, providing a template for antisense riboprobe protecting a 185 bp mRNA fragment. The IGF-II clone, constructed in the vector pBluescript II (Stratagene, La Jolla, CA) was linearized with BamHI, providing a template for antisense riboprobe protecting a 152 bp mRNA fragment. The rpL32 human cDNA clone (encoding ribosomal protein L32) [24] was provided by Dr R. Rochford (The Scripps Research Institute, La Jolla, CA). This recombinant constructed in a pGem 4 vector (Promega) was linearized with Eco IR, providing a template for antisense riboprobe protecting a 76 bp mRNA fragment.

RNAse protection assay

Total cellular RNA from HD and HI tumor specimens was isolated by the guanidinium thiocyanate–cesium chloride method [25]. Antisense ^{32}P-labeled RNA probes were transcribed (IGF-I and rpL32 with T7 RNA polymerase, IGF-II with T3 RNA polymerase) following manufacturer's protocol (Promega). Fifty μg of total RNA were hybridized with 2×10^5 cpm of each probe in the conditions described by Guerra *et al.* [20], followed by digestion with RNase A (Sigma). All RNA samples were hybridized with IGF-I or -II probes and rpL32 probe (used to correct for small variations in the amount of RNA loaded) simultaneously. Samples were extracted with phenol/chloroform/isoamyl alcohol (20:20:1) and precipitated with 20 μg of tRNA (Sigma) and 2 vol. of absolute ethanol. The pellets were resuspended in 5 μl of an 80% formamide loading buffer and run on a 6% polyacrylamide sequencing gel with 8 M urea. Size markers were prepared by end labeling Hinf I-digested fragments of PFH3 plasmid. Band intensities were quantitated by scanning the autoradiograms using a LKB (Bromma, Sweden) Ultroscan XL densitometer.

In situ hybridizations

Five micron frozen sections were cut and fixed in cold methanol, dried and stored at −80°C. The slides were allowed to come to room temperature and incubated with 2 × SSC (0.30 M NaCl, 0.030 M trisodium citrate, pH 7.0) for 30 min and then treated with 10–15 μg/ml proteinase-K in 20 mM Tris–HCl pH 7.4, 2 mM $CaCl_2$ for 15 min at 37°C. Enzyme solution was washed with PBS and the slides dehydrated in ethanol. The samples were incubated with 10 μl of the hybridization mixture consisting of 5×10^7 cpm/ml (^{35}S)UTP labeled antisense or sense IGF-I or -II RNA probes in 40% formamide, 4 × SSC, 0.1 M sodium phosphate buffer, pH 8 for 2 h at 37°C and then 18 h at room temperature under siliconized coverslips. The coverslips were removed after immersing the slides in 2 × SSC for 15 min, and then the samples were washed three times with PBS, dehydrated in graded alcohols and air dried. The slides were coated with photographic emulsion (Ilford K5), blot dried, dehydrated by placing them in an open black slide box in a light tight container with drierite, overnight, and then stored at −70°C for one month. The slides were developed in Kodak developer (D-19), immersed in 1% acetic acid, fixed using Kodak fixative, washed in running water, counterstained with hematoxylin, dehydrated, cleared and mounted.

RESULTS

IGF-I and IGF-II expression

Expression of IGF-II at protein level was detected in HD and HI tumor lines. A significant increase in IGF-II levels was found in HD tumors growing in MPA-treated animals, and in HI tumors, as compared with HD tumors growing in untreated animals (Table 1). IGF-II mRNA expression was studied using a RNAse protection assay. A three- to four-fold increase in IGF-II mRNA levels was detected in tumors growing in MPA-treated animals, as compared with untreated mice (Fig. 1(A)). IGF-II message levels in HI lines were similar to those observed in HD tumors growing in MPA-treated mice (Fig. 1(A)). Authentic IGF-I message was also detected in HD and HI lines. Neither MPA-induction

Table 1. *Quantification of IGF-II and Man-6P/type II IGF-R in MPA-induced mouse mammary adenocarcinomas*

	IGF-II (ng/g tissue)	Man 6P/type II IGF-R (fmol/mg prot)
	HD lines	
D5	91.2 ± 10.7 (4)	12,641 ± 2970 (3)
D5 + MPA	292.6 ± 35.7* (4)	6916 ± 840* (3)
C4	86.0 ± 11.5 (5)	12,827 ± 2808 (3)
C4 + MPA	346.8 ± 42.2* (4)	4214 ± 413* (3)
C7	65.2 ± 6.4 (3)	12,318 ± 2851 (3)
C7 + MPA	465.0 ± 57.2* (3)	6473 ± 760*(2)
	HI lines	
D5	371.8 ± 69.3* (3)	14,910 ± 1501 (3)
C4	426.9 ± 49.4* (7)	17,349 ± 2378 (3)
C7	531.2 ± 45.6* (3)	11,349 ± 2227 (3)

The amounts of IGF-II protein were calculated by a radio-receptor assay after acid-extraction and Sep-Pack C18 chromatography as described in Materials and Methods. The number of Man 6P/type II IGF-R was determined by using the IGF-II receptor binding assay described in Materials and Methods. Each value represents mean ± SD of (n) tumor samples.

+MPA: tumors growing in MPA-treated mice.

*$p < 0.001$ compared with the respective HD line growing in untreated mice by Student's t test.

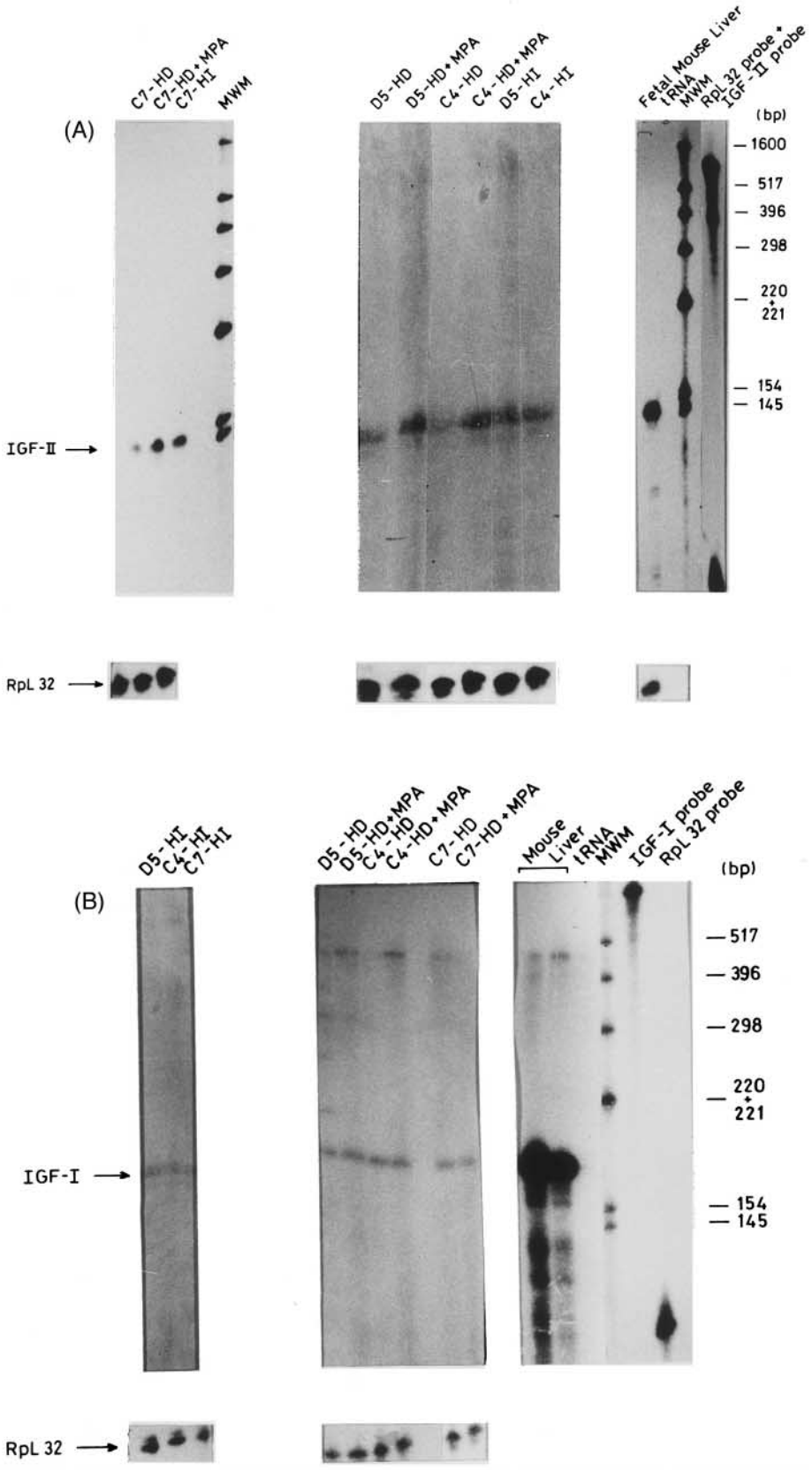

Fig. 1. IGF-I and -II expression in HD and HI tumor lines. IGF-II (A) and IGF-I (B) RNAse protection assay. Fifty μg of total RNA from the tumors were hybridized with IGF-I and IGF-II probes and rpL32 probe as described in Section 2. Total RNA from mouse liver was used as positive control for IGF-I expression and fetal mouse liver for IGF-II expression. The IGF-I protected fragment is 182 bp, the IGF-II protected fragment 152 bp, and the RpL32 protected fragment is 76 bp. MWM: molecular weight markers. +MPA: tumors growing in MPA-treated mice.

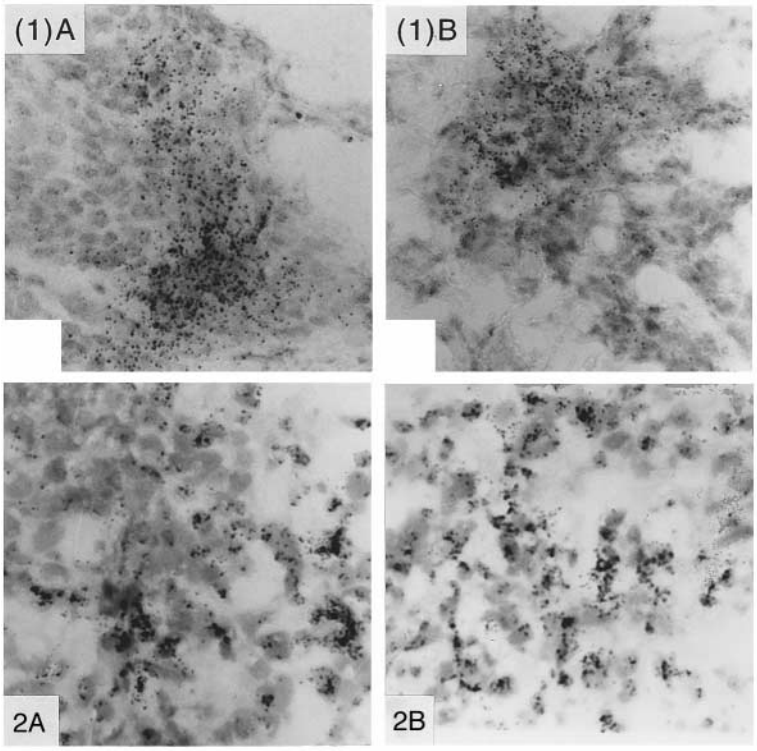

Fig. 2. Cellular localization of IGF-I and -II in HD and HI tumors. *In situ* hybridization of C4-HD (1) and C4-HI (2) tumors with IGF-I (A) and IGF-II probes (B). Hybridization signal is evident in the areas corresponding to the malignant epithelial tissue. Stroma is scanty and not very conspicuous in the pictures owing to the lack of cytoplasmic counterstaining.

of IGF-I message nor differences in IGF-I mRNA levels between HD and HI lines were found (Fig. 1(B)).

In situ hybridization studies

To identify the source of IGF-I and IGF-II mRNA in HD and HI lines, we examined tumor specimens by using *in situ* hybridization. Both IGFs gave strong positive signal in the areas corresponding to the malignant epithelial tissue in the HD and HI lines. No evident differences were seen between background and stromal staining. Sense probes failed to give any hybridization signal. Representative results in C4-HD and C4-HI tumors are shown in Fig. 2.

IGFs receptors

Specific IGF-II binding to tumor membranes was observed. A similar number of Man-6P/type II IGF-R was found in HD lines, growing in untreated mice, and in HI lines (Table 1). A significant decrease in Man-6P/type II IGF-R number was found in HD tumors growing in MPA-treated mice (Table 1). Chemical cross-linkings of ^{125}I-IGF-II to tumor membranes were performed. When analyzed by SDS polyacrylamide gel electrophoresis under reducing conditions, membrane preparations from HD and HI lines revealed a single labeled band of apparent MW 260 kDa, corresponding to the Man-6P/type II IGF-R. Figure 3 shows a representative cross-linking of ^{125}I-IGF-II to membranes from C4-HD and C4-HI lines. The specificity of these receptors was determined by studying the relative potencies of unlabeled

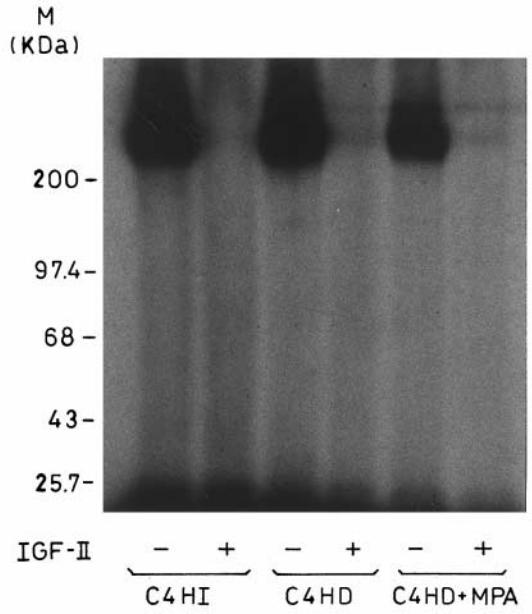

Fig. 3. Chemical cross-linking of ^{125}I-IGF-II to membrane preparations of C4-HI and C4-HD tumors growing in MPA-treated and untreated mice. IGF-II was affinity cross-linked to its receptor in the absence (–) or the presence (+) of an excess of 500 ng unlabeled IGF-II. M: molecular weight markers.

IGF-I, IGF-II, and insulin to competitively inhibit ^{125}I-IGF-II binding in HD and HI tumor membranes. Figure 4(A) shows the competition for ^{125}I-IGF-II binding to C4-HD line membranes. Unlabeled IGF-II was able to completely inhibit ^{125}I-IGF-II binding in a dose-dependent manner, with a half maximal displacement at a concentration of 2 nM. IGF-I 80 nM inhibited 40% of the ^{125}I-IGF-II binding, whereas same concentration of insulin was not effective. IGF-I, IGF-II and insulin exhibited similar relative potencies as competitive inhibitors of ^{125}I-IGF-II binding in the other HD and HI lines (data not shown). As we had found previously [3] that type I IGF-R levels were similar in HD and HI lines, we further characterized IGFs binding to the type I IGF-R present in our experimental tumors. The relative potencies of unlabeled IGF-I, IGF-II, and insulin to competitively inhibit ^{125}I-IGF-I binding in HD and HI tumor membranes were determined. In competition studies for ^{125}I-IGF-I binding to C4-HD line membranes, half maximal inhibition was obtained with 1.25 nM of IGF-I and with 71 nM IGF-II. Insulin at a concentration of 60 nM inhibited only 30% of ^{125}I-IGF-I binding (Fig. 4(B)); IGF-I, IGF-II and insulin exhibited similar relative potencies as competitive inhibitors of ^{125}I-IGF-I binding in the other HD and HI lines evaluated (data not shown).

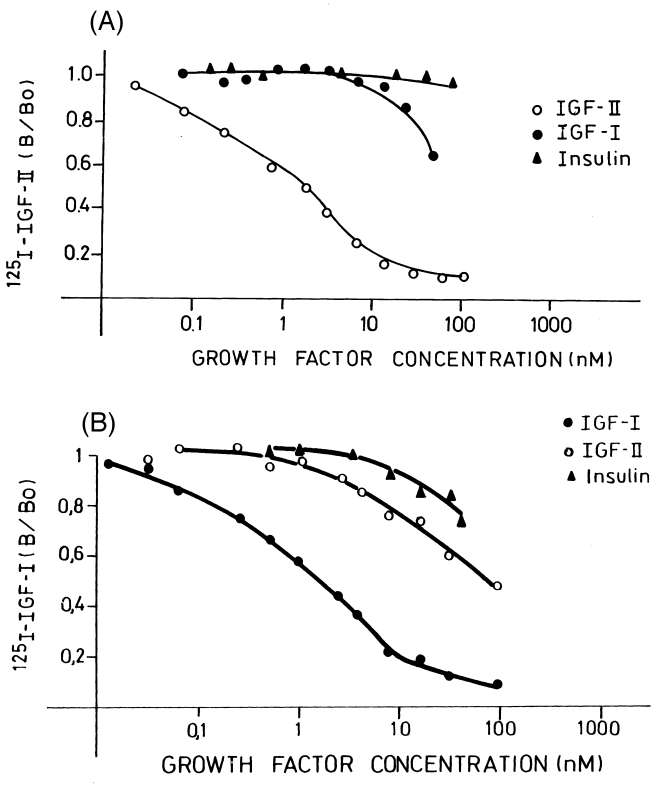

Fig. 4. Competitive inhibition of ^{125}I-IGFs binding to C4-HD tumor membranes. Thirty μg protein from C4-HD microsomal membranes were incubated with 50,000 dpm ^{125}I-IGF-II (A) or ^{125}I-IGF-I (B) and increasing concentrations of unlabeled IGF-I (●), IGF-II (○) and insulin (▲). The data are plotted as B/B_0, i.e. ligand bound in the presence of competing unlabeled ligand, divided by the quantity of ligand bound in the absence of competition, after subtracting nonspecific binding. Figures represent the mean of three separate experiments.

Involvement of IGFs in the proliferation of primary cultures of C4-HD tumor line

C4-HD tumor line was selected to study IGFs involvement in *in vitro* growth because it has exhaustively been characterized as regard to its *in vitro* proliferative behavior and response to MPA [6].

Since we found that the malignant epithelial cells in HD and HI tumors expressed IGF-I and -II, we first investigated the cellular localization of IGFs receptors to then evaluate whether autocrine pathways involving IGFs could participate in MPA-induced growth. IGFs binding was studied in primary cultures of epithelial cells from C4-HD tumors. Cross-linking studies performed with ^{125}I-IGF-I and IGF-II under reducing conditions demonstrated that type I and Man-6P/type II IGF-R were expressed in epithelial cells (Fig. 5).

Effects of exogenous IGF-I and -II in the proliferation of epithelial cultures of C4-HD tumor line

IGF-I at concentrations ranging from 5 to 100 ng/ml was added to C4-HD epithelial cells and incorporation of (^{3}H)-thymidine was used as a measure of DNA synthesis and mitogenic activity. Although IGF-I alone did not stimulate cell proliferation, it was able to potenciate MPA stimulatory effect at concentrations of 50–100 ng/ml (Fig. 6). Exogenously added IGF-II at concentrations ranging from 5 to 100 ng/ml did neither increase (^{3}H)-thymidine incorporation to C4-HD epithelial cells nor potentiate MPA mitogenic effect (data not shown).

Antisense studies

Incubation of C4-HD epithelial cells with antisense oligodeoxynucleotides (ASODNs) to type I IGF-R RNA resulted in a dose-dependent inhibition of MPA-mediated cell proliferation. Treatment of C4-HD cells with 40 μg/ml ASODNs completely inhibited MPA-induced cell growth. (^{3}H)thymidine uptake at this concentration was significantly lower than in control cells growing in 1% steroid-stripped fetal calf serum (ChFCS) (Fig. 7(A)). Sense ODNs (SODNs) at concentrations up to 40 μg/ml had no effect on cell growth (Fig. 7(A)). There was no evidence of toxicity in response to ODNs as determined by loss of cells into the medium (data not shown). The effect of type I IGF-R ODNs on type I IGF-R number was assessed by performing binding studies of IGF-I on washed membranes from C4-HD cells. Cells treated with 40 μg/ml of ASODNs showed a 75% decrease in the number of type I IGF-R as measured by Scatchard plot analysis [22]. The number of receptors in control or SODNs-treated C4-HD epithelial cells

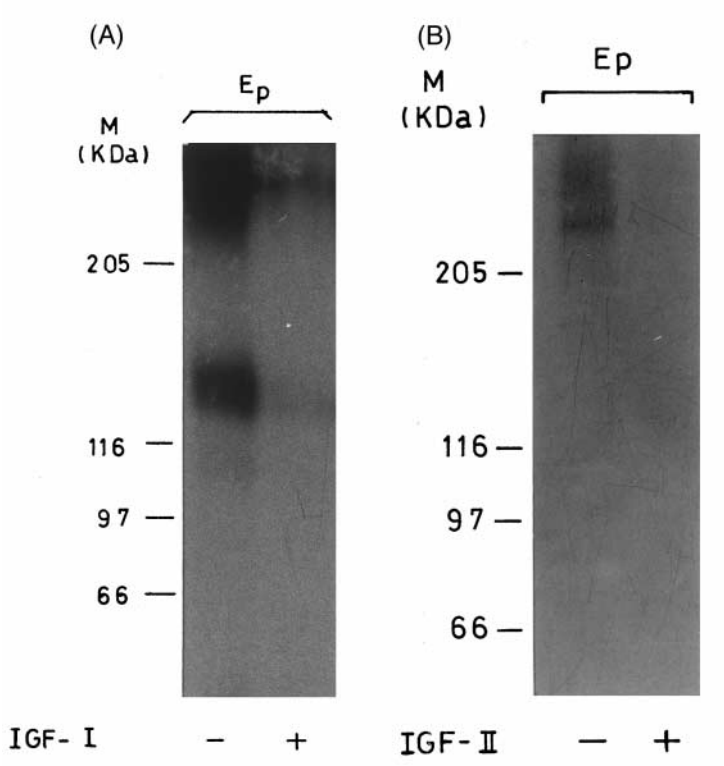

Fig. 5. IGF-I and -II binding in monolayer cultures of epithelial cells from C4-HD tumors. Cross-linking studies were performed with ^{125}I-IGF-I (A) and IGF-II (B) under reducing conditions as described in Materials and Methods.

was 53.1 ± 3.5 fmol/mg protein, while in cells treated with the ASODNs it was 13.6 ± 1.3 fmol/mg protein.

Our findings showing MPA-up regulation of IGF-II levels in HD tumor lines, suggest a role for IGF-II in MPA-mediated growth. To further evaluate this hypothesis, epithelial primary cultures of C4-HD tumors were incubated with IGF-II ASODNs. A concentration-dependent inhibition of MPA-induced cell proliferation of C4-HD cells was seen with ASODNs (Fig. 7(B)). The control sense ODN or the scrambled sequence ASODN had no significant effect on cell growth (Fig. 7(B)). Inhibition caused by IGF-II ASODNs could not be overcome by the addition of up to 150 ng/ml of human recombinant IGF-II to the medium. The effect of IGF-II ASODNs on IGF-II synthesis was assessed by immunoblotting C4-HD cells lysates using a polyclonal antibody raised against a peptide mapping at the carboxyl terminus of mouse

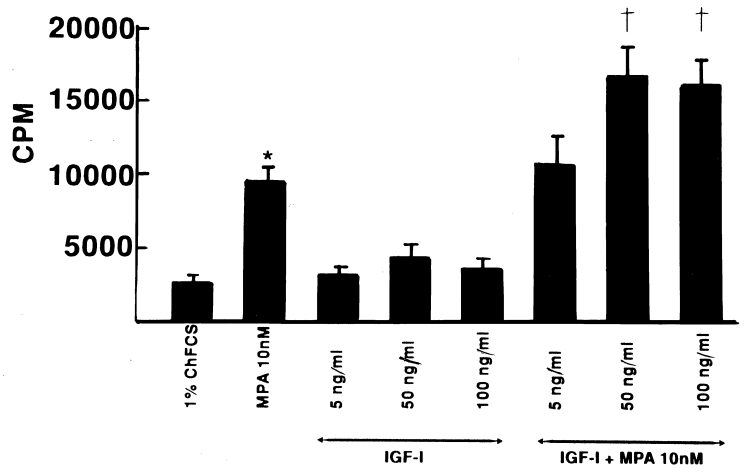

Fig. 6. Effects of IGF-I on the proliferation of C4-HD epithelial cells. Primary cultures of epithelial cells from C4-HD tumors were incubated in medium with 1% ChFCS, MPA 10 nM, IGF-I at various concentrations, or MPA + IGF-I. Incorporation of (^{3}H)-thymidine was used as a measure of DNA synthesis. *$p < 0.001$ vs cells growing in 1% ChFCS, †$p < 0.05$ vs MPA 10 nM. Data are presented as mean ± SD. The experiment shown is representative of a total of four.

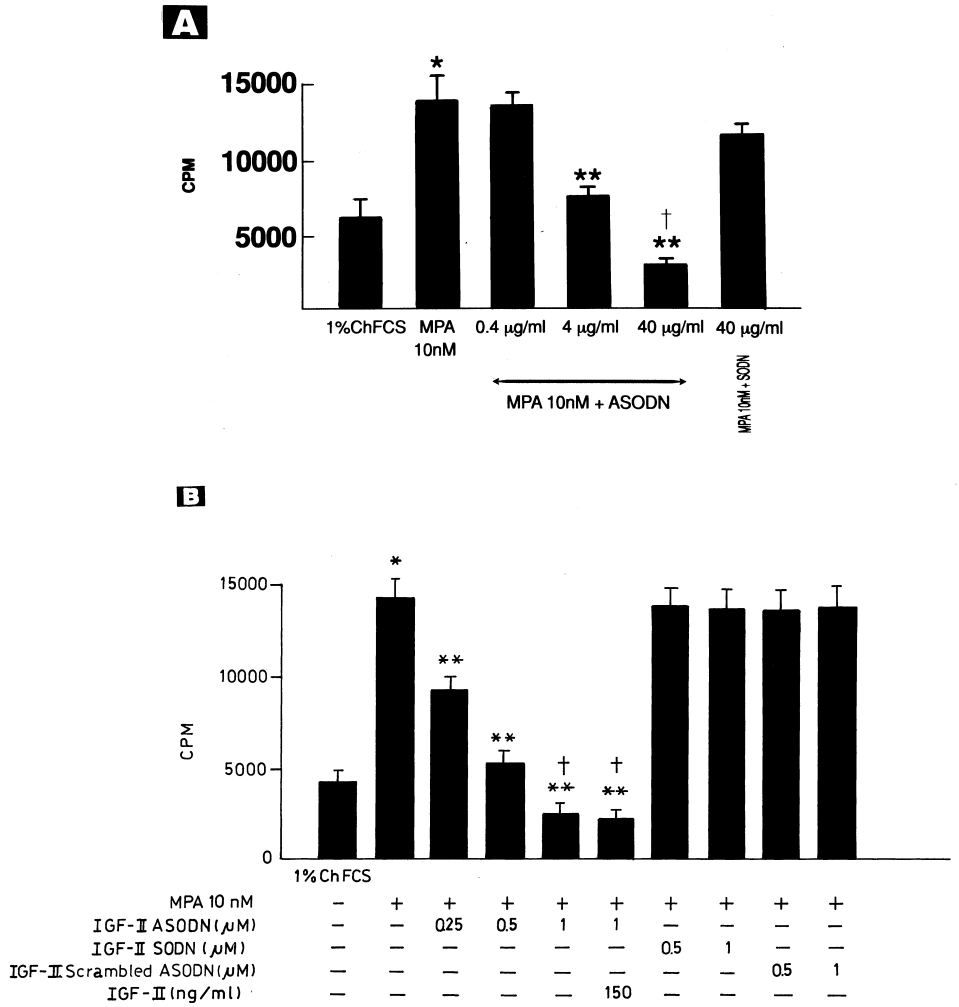

Fig. 7. Inhibition of C4-HD epithelial cells proliferation by ASODNs to type I IGF-R RNA (A) and to IGF-II (B). C4-HD cells were incubated for 48 h in medium with 1% ChFCS, MPA 10 nM, MPA 10 nM + various concentrations of ASODNs to type I IGF-R RNA or to IGF-II RNA, and MPA 10 nM + the indicated concentrations of SODNs. In the case of IGF-II, cells were also incubated with an scrambled ASODN and with MPA 10 nM + 1 μM ASODN with the addition of 150 ng/ml of IGF-II. (^{3}H)thymidine incorporation into DNA was determined. Data are presented as mean ± SD. *, † $p < 0.001$ vs cells growing in 1% ChFCS; **$p < 0.001$ vs MPA 10 nM. The experiments shown are representative of a total of four performed for type I IGF-R ASODNs and of three performed for IGF-II ASODNs.

IGF-II precursor. During the processing of prepro-IGF-II to the mature 7 kDa IGF-II, carboxy-terminal residues get cleaved [26]. Thus, this antibody cannot recognize the mature IGF-II or forms in which carboxyl terminus have been cleaved. A protein of approximately 14 kDa, the size expected for partially processed pro-IGF-II, was detected in control cells treated with MPA or MPA + ODNs (Fig. 8). Densitometric evaluation demonstrated that IGF-II levels were reduced by 83% after 1 μM ASODNs treatment, while SODNs did not reduce IGF-II levels.

DISCUSSION

In this work, we have demonstrated an *in vivo* MPA up-regulation of IGF-II mRNA and protein levels in HD tumor lines. Progression to a hormone-independent phenotype in all these lines was accompanied by a high constitutive expression of IGF-II. Authentic IGF-I message was also found in HD and HI tumors. As we have previously reported in the study of IGF-I protein, similar IGF-I mRNA levels were found in HD and HI lines. Our *in situ* hybridization studies demonstrated that IGF-I message arose from the malignant epithelial cells in HD and HI tumors. This finding provides additional evidence to recent reports indicating IGF-I immunohistochemical localization in the epithelial cells of human breast carcinomas, enabling its function as an autocrine GF [27]. The role of IGF-I as an autocrine or paracrine growth factor for breast tumor epithelial cells remains controversial, since it is strongly mitogenic for breast tumor epithelial cells but, in a number of studies of IGF-I expression, positive signals were detected only in stromal cells [28, 29]. IGF-II mRNA

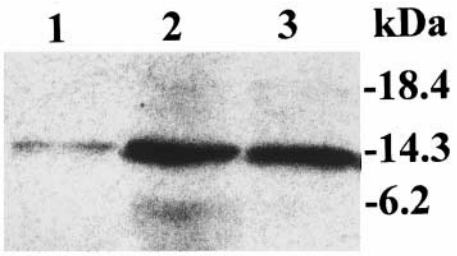

Fig. 8. Effect of IGF-II ASODNs on IGF-II synthesis. C4-HD cell lysates were prepared as described in Materials and Methods and 80 μg of protein extracts were electrophoresed and immunoblotted for IGF-II. C4HD cells growing in 10 nM MPA + 1 μM ASODN (1), in 10 nM MPA + 1 μM SODN (2), and in 10 nM MPA (3). Densitometric analysis of IGF-II band expressed as a percentage of the control value (i.e. C4HD cells growing in 10 nM MPA) is: 17% for cells treated with 1 μM ASODN. No significant differences in densitometric values of IGF-II bands between control cells and cells treated with 1 μM SODN were found. This is an autoradiogram from a representative experiment of a total of three in which SE was within 10%.

expression was present only in the malignant epithelial cells of our HD and HI tumors. In breast tumor specimens, IGF-II mRNA have been found in both malignant epithelial cells and in the adjacent stroma [29]. In addition, IGF-II message has been detected in several breast cancer cell lines [11, 12]. Interactions between progestin and IGFs were reported in experimental mammary tumors. Manni *et al.* [18] demonstrated that progesterone (Pg) stimulation of soft-agar growth in the hormone-responsive *N*-nitrosomethylurea (NMU)-rat mammary tumor was accompanied by production of IGFs-related peptides, and that anti-IGF-I antibodies inhibited Pg-stimulated growth. The same group [17] also showed that Pg stimulation of MCF-7 cells soft-agar growth was inhibited by anti-IGF-I and type I IGF-R antibodies. Progestins were also found to increase IGF-II expression in T-47D cells [16] although, unlike our findings, progestins enhanced IGF-II protein synthesis with no effect on IGF-II mRNA [16].

Two patterns of relationships between IGF-II and Man-6P/type II IGF-R content were found in our tumor model. Tumors from HD lines growing in MPA-treated mice showed high IGF-II expression and low Man-6P/type II IGF-R content, as compared with HD tumors growing in untreated mice. On the other hand, in HI tumors we found high IGF-II expression with high Man-6P/type II IGF-R levels. A possible explanation for the decrease in Man-6P/type II IGF-R in HD tumors growing in MPA-treated mice could be an MPA direct negative regulation of the Man-6P/type II IGF-R transcription/translation. Further support to this hypothesis was provided by the fact that in HI tumors, notwithstanding high IGF-II synthesis, the levels of Man-6P/type II IGF-R remain high. A correlation between high IGF-II content and low Man-6P/type II IGF-R concentration had already been found in breast cancer [30]. In addition, a new role as a tumor suppressor gene has been postulated for Man-6P/type II IGF-R in recent reports, showing that when IGF-II binds to this receptor, it gets internalized and degraded turning unavailable to activate the type I IGF-R signaling [31–34]. From that perspective, the decrease in Man-6-P/type II IGF-R could result in inhibition of IGF-II degradation and in consequent accumulation of IGF-II available to activate the type I IGF-R. The function of Man-6P/type II IGF-R remains to be elucidated. It has been reported to possibly play a role in stimulating cellular responses such as proliferation and motility [35, 36]. However, the hypothesis that Man-6P/type II IGF-R mediates mitogenic actions of IGF-II through specific activation of G proteins has been challenged [37]. In our HD and HI lines, competitive inhibition and affinity labeling studies showed an almost exclusive binding of recombinant IGF-II to Man-6P/type II IGF-R, suggesting that IGF-II effects could be mediated by binding to this receptor. Man-6P/type II IGF-R involvement in IGF-II-mediated growth has also been suggested in MCF-7 cells and in T61 and MCF-7 breast cancer xenografts, where the anti-type I IGF-R antibody αIR3 cannot completely block IGF-II mitogenic effects [38–40]. However, in many model systems, type I IGF-R appears to be responsible for mediating the mitogenic effects of both IGF-I and -II [41, 42]. MPA independent growth does not involve a decrease in the number of Man-6P/type II IGF-R, suggesting that this receptor does not play a role as a tumor suppressor in HI tumors, but could mediate biological functions other than those related to proliferation. It has been described that overexpression of IGF-II in MCF-7 cells, possibly acting through Man-6P/type II IGF-R, induced phenotypic changes associated with tumor progression, that are not simply the result of mitogenic stimuli [15].

The *in vitro* studies of the effects of exogenous IGF-I in the proliferation of primary cultures of C4-HD epithelial cells showed that supplementation of C4HD cells with concentrations up to 100 ng/ml IGF-I had no effect on DNA synthesis. However, exogenous IGF-I potentiates MPA stimulatory effect on C4-HD epithelial cells growth. This could be due to an IGF-I increase in PR protein level, as previously reported in MCF-7 breast cancer cells [19]. Alternatively, signal transduction pathways activated by IGF-I might modulate PR function through phosphorylation, as described for estrogen receptor (ER) in rat uterus [43].

We had formerly found similar levels of type I IGF-R in HD and HI tumor lines [3]. In this research, cellular localization studies demonstrated that this receptor is present in epithelial cells from C4-HD tumors. Furthermore, the different affinities for IGF-I and -II exhibited by the type I IGF-R

found in our tumors, suggest the presence of an atypical receptor. Type I IGF-R plays a central role in the establishment and maintenance of the transformed phenotype for several cell types both *in vitro* and in intact animals [41, 44–47]. Furthermore, antisense strategies against type I IGF-R RNA show that the decrease in the number of type I IGF-R causes a reversal of the transformed phenotype in a variety of cell types (reviewed in [48]). Particularly in breast cancer, stable expression of type I IGF-R antisense RNA in MCF-7 cells correlated with a significant decrease in IGF-I- and serum-stimulated growth [49]. In the present report, we demonstrated by using an antisense strategy to type IGF-R mRNA that this receptor participates in MPA-induced mitogenesis of HD mammary epithelial cells. Furthermore, the finding that (^{3}H)thymidine uptake at 40 μg/ml concentration of type I IGF-R ASODN was significantly lower than in control cells growing in 1% ChFCS, strongly supports the existence of an autocrine mechanism to stimulate cell growth in C4-HD cells, depending on IGF-I and type I IGF-R. In addition, constitutive activation of this autocrine loop, involving IGF-I and type I IGF-R, could be necessary for MPA-induced IGF-II enhancement of HD cells proliferation.

The results obtained with IGF-II ASODNs demonstrate that IGF-II also participates in MPA induced mitogenesis of HD mammary epithelial cells. Though C4-HD cells do not respond to supplemental IGF-II, blocking endogenous IGF-II by ASODNs inhibited MPA-induced cell growth. Furthermore, the inhibition caused by IGF-II ASODNs could not be overcome by the addition of human recombinant IGF-II to the medium. These findings led us to hypothesize that IGF-II synthesized by C4-HD cells could act by an intracrine mechanism to stimulate DNA synthesis. IGF-I intracrine actions were suggested in lung fibroblast cell line WI-38, in which exogenous IGF-I had no significant effect on DNA synthesis, but IGF-I ASODNs inhibited DNA synthesis [50]. Inactivation of exogenous IGF-II by IGF-BP secreted by C4-HD cells could be another cause of its failure to stimulate DNA synthesis and to overcome IGF-II ASODNs inhibitory effects. In fact, we had formerly found the presence of IGF-BP in HD and HI MPA-induced mammary adenocarcinomas [3]. Again, intracrine interactions between endogenous IGF-II and its receptor in C4-HD cells could prevent IGF-II neutralization by secreted IGF-BPs. Finally, increased biological activity of the large 14 kDa IGF-II form found in C4-HD cells, reduced affinity for IGF-BPs, or affinity for different IGF-BPs could explain the results obtained with exogenous IGF-II [51].

In summary, our studies demonstrating expression of both IGF-I and type I IGF-R in tumor epithelial cells suggest that IGF-I could play an autocrine role in breast cancer, besides its well known paracrine role. High proliferative tumor rates typical of MPA-stimulated HD lines and of HI lines were associated with high levels of IGF-II expression. Interestingly, IGF-II seems to bind only to Man 6P/type II IGF-R in HD and HI tumors. This finding together with the direct evidence of the involvement of IGF-II as mediator of MPA-induced growth, provided by the ASODNs studies, adds further support for a possible role of Man 6P/type II IGF-R in IGF-II mitogenic actions. Finally, we have demonstrated that the blocking of type I IGF-R expression using an antisense strategy results in the inhibition of MPA-induced growth of mammary cancer cells.

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