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# Inhibition of Growth of Prostatic Cancer Cell Lines by Peptide Analogues of Insulin-like Growth Factor 1[1]

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## ABSTRACT

**We have investigated three prostatic cancer cell lines, PC-3, DU-145, and LNCa.FGC, and found that all three cell lines can grow in serum-free medium without the addition of exogenous growth factors. All three cell lines produce substantial amounts of insulin-like growth factor 1 (IGF-1) that is secreted in the medium and they all display constitutively autophosphorylated IGF-1 receptors; two of the cell lines overexpress IGF-1 receptor RNA. The growth of all three cell lines is inhibited by an antisense oligodeoxynucleotide to IGF-1 receptor RNA or by peptide analogues of IGF-1 that compete with IGF-1 for binding to its receptor. Our results indicate that these three cell lines grow by an autocrine loop in which the overproduced IGF-1 activates its receptor. Interference with the activation of the receptor leads to cessation of growth.**

## INTRODUCTION

The activation of the IGF-1[3] receptor by its ligands (IGF-1, IGF-2, or insulin at supraphysiological concentrations) seems to play a pivotal role in the proliferation of a variety of cell types, such as fibroblasts, smooth muscle cells, chondrocytes, hemopoietic cells, and others (for a review, see Ref. 1). Recently, we have shown that 3T3 cells constitutively overexpressing the human IGF-1 receptor grow in serum-free medium supplemented solely with IGF-1, to the total exclusion of the receptors for the other 2 growth factors that are required by 3T3 cells, platelet-derived growth factor and EGF (2). The importance of the IGF-1/IGF-1 receptor interaction in cell proliferation was confirmed by other findings from our laboratory: (*a*) overexpression of c-*myb* abrogates the requirement for IGF-1 in 3T3 cells, by inducing an increase in the expression of IGF-1 itself (3); (*b*) EGF markedly increases the expression of IGF-1 and IGF-1 mRNA, and growth stimulation by EGF indirectly requires a functional IGF-1 receptor (4); and (*c*) the SV40 large T-antigen also markedly increases the expression of IGF-1 and IGF-1 mRNA, leading to reduced growth factor requirements for SV40-transformed cells (5). In a recent review, Macaulay (6) has summarized the evidence indicating the role played by IGF-1, IGF-2, and their receptors in the growth of a variety of human tumors. Because the IGF-1/IGF-1 receptor autocrine loop occurs frequently in different types of tumors, we thought it desirable to determine whether IGF-1 was required for the growth of prostatic cancer cell lines in culture. Our results show that the 3 prostatic cancer cell lines examined grow in serum-free medium, produce abundant amounts of IGF-1, and require an activated IGF-1 receptor for growth.

## MATERIALS AND METHODS

**Cell Lines.** The following cell lines were obtained from the American Type Culture Collection: PC-3 (7) originating from a human adenocarcinoma of the prostate; LNCa.FGC (LNC; see Ref. 8) from a human metastatic adenocarcinoma of the prostate; and DU-145 (9) from a human carcinoma of the prostate metastatic to the brain. The cells were passaged as recommended by the American Type Culture Collection; they are now grown in the following serum-free medium: DU-145 in minimum essential medium supplemented with 1 μM ferrous sulfate; 1 mM sodium pyruvate and 0.1% bovine serum albumin. The same supplements were added to Dulbecco's modified Eagle's medium:RPMI 1640 (1:1) to grow PC-3 cells and to RPMI 1640 for LNC cells.

**Growth in Serum-free Medium.** For these experiments, the cells were plated first in 10% calf serum; the growth medium was removed after 24 h and replaced with serum-free medium, with the sole additions of bovine serum albumin (0.5 mg/ml) and 1.0 μM ferrous sulfate (2). The number of cells was determined by standard methods at the times indicated in each individual experiment.

**RT-PCR.** We followed the method of Rappolee *et al.* (10), with the slight modifications used in our laboratory (11). RNA was extracted from cells using the method of Chomczynski and Sacchi (12). Amplimers and probe for the IGF-1 receptor RNA were chosen on the basis of the published complementary DNA sequence of the human IGF-1 receptor (13): 5′ amplimer, 5′ ACC ATT GAT TCT GTT ACT TC 3′; 3′ amplimer, 5′ ATA CTC TGT GAC ATT CTT AA 3′; probe 5′ CTG CTC CTC TCC TAG GAT GA 3′. Labeling of probes and hybridization were carried out by standard methods (14, 15). The various controls we use in our RT-PCR assays (elimination of DNA, rejection of samples that give signals without reverse transcriptase, and multiple amplification cycles) have been repeatedly described in articles from our laboratory (2, 4, 16). RNA amounts were monitored with amplimers and probe for the pHE 7 complementary DNA, the cognate RNA of which is expressed constantly under different conditions of growth (16).

**Antisense Experiments.** The antisense and sense oligodeoxynucleotides corresponded to codons 21–29 of the signal sequence of the human IGF-1 receptor (13). The actual sequences are given in the article by Pietrzkowski *et al.* (2) and the antisense oligodeoxynucleotide has already been shown to inhibit cell growth and to decrease the number of IGF-1 binding sites (2, 4). The oligodeoxynucleotides were synthesized on an Applied Biosystem Model 391 EP DNA synthesizer as already described (2, 4, 16). The oligodeoxynucleotides were added to the medium after 48 h in serum-free medium (40 μg/ml) and the treatment was repeated the next day (20 μg/ml). The cells were counted 48 h after the second addition.

**Autophosphorylation of the IGF-1 Receptor.** This was carried out essentially by the method of Lammers *et al.* (17), using the monoclonal antibody to the IGF-1 receptor from Oncogene Sciences (Uniondale, NY), an anti-phosphotyrosine antibody from UBI (Saranac Lake, NY), and the enhanced chemiluminescence detection system from Amersham (Arlington Heights, IL).

**IGF-1 Radioimmunoassay.** This has been described in detail in a previous paper (4), following the instructions of the manufacturer of the kit (Amersham, Arlington Heights, IL).

**IGF-1 Peptide Analogues.** The IGF-1 peptide analogues used in these experiments have been described in a paper by Pietrzkowski *et al.* (18). They inhibit autophosphorylation of the IGF-1 receptor by IGF-1 and inhibit the growth of cells that require IGF-1 for growth.

## RESULTS

**Growth Requirements of Cell Lines.** The three prostatic cancer cell lines we obtained from American Type Culture Collection were originally passaged as recommended by the source. However, in order to determine the growth factors requirements of the cell lines, we tested them for their ability to grow in serum-free medium (see "Materials and Methods"). The results are shown in Fig. 1, from which it is evident that all three cell lines, DU-145, PC-3, and LNC grow in

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[3] The abbreviations used are: IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; RT-PRC, reverse-transcriptase polymerase chain reaction.

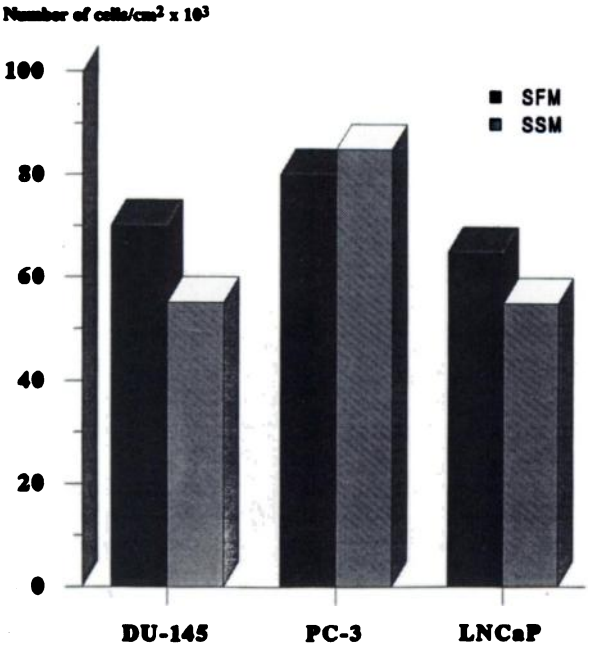


Fig. 1. Growth of prostatic cancer cell lines in serum-free medium. *Abscissa*, the 3 cell lines used. In all cases, the cells were plated at a concentration of $10 \times 10^3$ cells/cm$^2$ in serum-supplemented medium. After 24 h, one-half of the plates were changed to serum-free medium (*SFM*), while the other half was kept in the original growth medium (*SSM*). The number of cells (*ordinate*) was determined after 72 h.

serum-free medium as vigorously as in serum-supplemented medium. It is true that the cells were plated in serum-supplemented medium for 24 h, but the medium is then removed carefully by repeated washings, and, under the same conditions, neither 3T3 nor p6 cells nor human diploid fibroblasts grow at all (4). The serum used for plating serves actually as an attachment factor and can be substituted with poly-L-lysine (4).

We then tested the effect of individual growth factors on the growth of the three prostatic cancer cell lines. The results of such an experiment are shown in Fig. 2. None of the added growth factors (EGF, platelet-derived growth factor, or IGF-1) increased the growth of the DU-145 and PC-3 cell lines, when compared to serum-free medium (Fig. 2, *Bars 2–5*). With LNC cells, they actually showed a slight inhibitory effect.

**An Antisense Oligodeoxynucleotide to IGF-1 Receptor RNA Inhibits the Proliferation of Prostatic Cancer Cell Lines.** In a previous article (2), we had a shown that 3T3 cells stably transfected with and constitutively overexpressing the IGF-1 and IGF-1 receptor RNA grew in serum-free medium to the total exclusion of added exogenous growth factors. We showed that these cells, called p12 cells, had established an autocrine mechanism that could be interrupted only by incubating the cells with an antisense oligodeoxynucleotide to the IGF-1 receptor RNA. We asked whether a similar mechanism may exist in the three prostatic cancer cell lines. Fig. 3 shows that, indeed, an antisense oligodeoxynucleotide to IGF-1 receptor RNA effectively inhibits the growth of all three cell lines, while a sense oligo has no appreciable effect. We have documented in previous articles the specificity of the antisense oligo used and its ability to decrease the levels of IGF-1 receptor protein (2, 4, 5, 16).

We also tested these cells for their ability to express IGF-1 receptor RNA, and the results (by RT-PCR) are shown in Fig. 4, where we also included RNAs from p12 cells (which constitutively overexpress IGF-1 receptor RNA; see above) and WI-38 human diploid fibroblasts, as an example of a normal cell in culture that requires IGF-1 for growth (19). Two of the cell lines, PC-3 and DU-145, express levels of IGF-1 receptor RNA that are about 10-fold (by densitometry) that of the levels in WI-38 cells. LNC cells express levels that are only slightly above those of WI-38. The amounts of RNA in each reaction (monitored as described in "Materials and Methods") were within 10% of each other (data not shown). We therefore characterized these cell lines for levels of IGF-1 receptor that can be autophosphorylated by IGF-1 and for the ability to produce and secrete IGF-1 in the medium. Autophosphorylation of the IGF-1 receptor was determined as described in "Materials and Methods," and the results are shown in Fig. 5. Although the amount of autophosphorylated IGF-1 receptor is not as high as in p12 cells (Fig. 5, *Lane 1*), which constitutively overexpress the human IGF-1 receptor (2), substantial amounts of receptor that can be autophosphorylated by IGF-1 are detectable in all cell lines (Fig. 5, *Lanes 2–4*). More significantly, the autophosphorylation of the receptor can be detected in cells growing in serum-free medium even without the addition of IGF-1 (Fig. 5, *Lanes 5–7*), indicating that, in these cell lines, the IGF-1 receptor is constitutively autophosphorylated, and that, presumably, this is due to the presence of measurable amounts of IGF-1, secreted by the cells themselves into the medium.

The amount of IGF-1 secreted in the medium was determined by a radioimmunoassay: the results (Table 1) are expressed as ng of IGF-1/ml/2 $\times$ 10$^6$ cells. All three cell lines are good producers of IGF-1, especially PC-3 cells. In all instances, however, the concentration of IGF-1 is more than sufficient to autophosphorylate the IGF-1 receptor and to sustain growth if the number of IGF-1 receptors is adequate (see above). Ordinarily, in cells expressing an adequate number of IGF-1 receptors, like p6 cells, 3 ng/ml IGF-1 are sufficient to induce autophosphorylation of the receptor and stimulation of growth (5).

We have confirmed that these cells require the IGF-1/IGF-1 receptor interaction by using peptide analogues of IGF-1 that completely inhibit autophosphorylation of the receptor by IGF-1 and growth of all

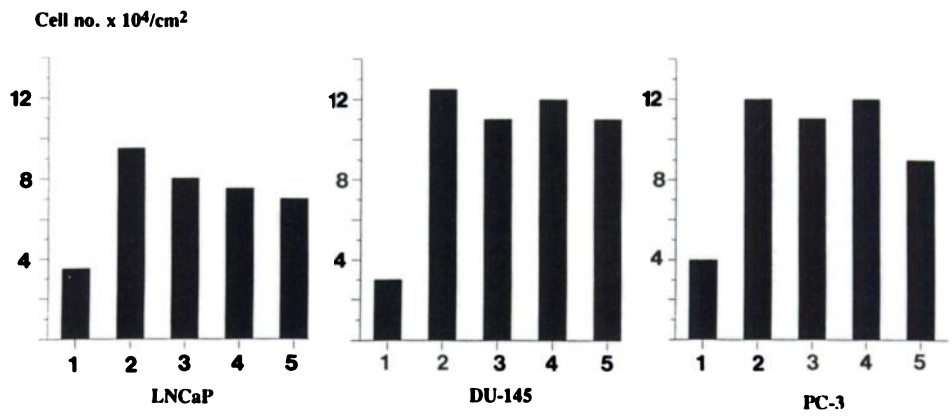


Fig. 2. Effect of individual growth factors on the growth of prostatic cancer cell lines. Cell lines (*abscissa*) were plated as in Fig. 1 and placed in serum-free medium after 24 h. After another 24 h, growth factors were added and the number of cells (*ordinate*) was determined 48 h after addition of growth factors (or a total of 96 h after plating). *Bar 1*, 48 h after plating; *Bar 2*, no additions; *Bar 3*, EGF (20 ng/ml); *Bar 4*, IGF-1 (20 ng/ml); *Bar 5*, platelet-derived growth factor (1 ng/ml). *Bars 2–5*, 96 h after plating.

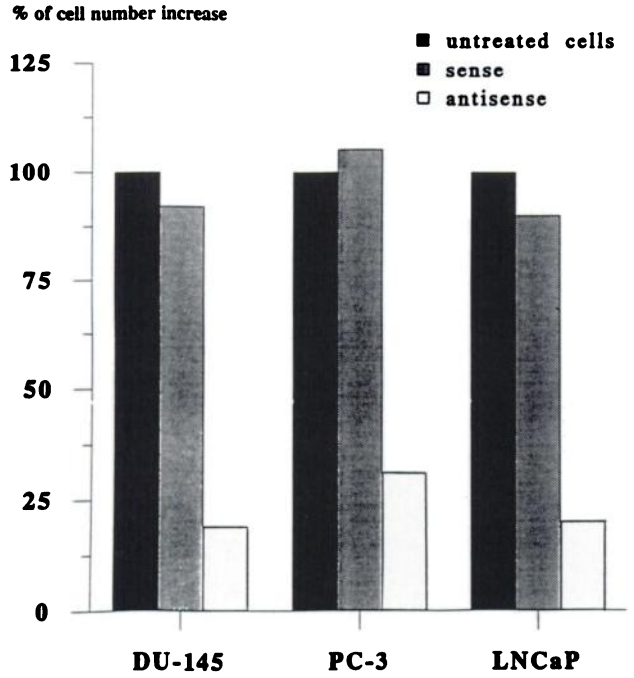


Fig. 3. Effect of an antisense oligodeoxynucleotide to the IGF-1 receptor RNA on the growth of prostatic cancer cell lines. Cell lines (*abscissa*) prepared to grow in serum-free medium as in Figs. 1 and 2 were incubated with either sense or antisense oligodeoxynucleotides to the IGF-1 receptor RNA as described in "Materials and Methods." The cells were counted 48 h after the second treatment with oligodeoxynucleotides. *Ordinate*, percentage increase in the number of cells over the number of cells at the time of the first addition of the oligodeoxynucleotides.

been growing *in vitro* for long periods of time. Still, there are many similar cell lines that still require growth factors or small amounts of serum for growth, as, for instance, HeLa cells. It is therefore reasonable to assume that, when cells *in vitro* grow in the complete absence of growth factors, it is because they produce their own growth factors. Recently, we have demonstrated that 3T3 cells constitutively overexpressing the IGF-1 and IGF-1 receptor RNAs (p12 cells) grow in serum-free medium, without any added exogenous growth factor. If the same 3T3 cells overexpress only the IGF-1 receptor RNA, they require only IGF-1 for growth (2). We therefore asked whether a similar mechanism may be operative in our 3 prostatic cancer cell lines.

Indeed, the same mechanism seems to operate in these cell lines, since all three produce substantial amounts of IGF-1 that is secreted in the medium. The concentrations given in Table 1 are quite high, when one considers that, in cells overexpressing the IGF-1 receptor, the addition of 3 ng/ml of IGF-1 in a single dose is sufficient to stimulate maximally cell proliferation. The concentration of IGF-1 in the medium was determined by radioimmunoassay, after eliminating the IGF-1 binding proteins (4). Our results do not contradict the recent report by Rossi and Zetter (22), who found that transferrin stimulated the growth of PC-3 and DU 145 cells in 0.25% fetal bovine serum. Our serum-free medium contains ferrous sulfate (2), which can substitute for transferrin in many cell types.

The production of IGF-1 is accompanied by a modest overexpression of the IGF-1 receptor and, at least in 2 cell lines, of the IGF-1 receptor RNA. It should be noted that variations in the number of IGF-1 binding sites are usually within narrow ranges (23, 24).

cell types that require IGF-1 for growth (18). Fig. 6 shows the effect of one of these peptides, which we call JB1, on the growth of the prostatic cancer cell lines. All three cell lines are inhibited in a concentration-dependent manner by the JB1 peptide, except for LNC cells, in which the highest concentration, 1 μg/ml, is slightly less effective than 500 ng/ml. We do not have any explanation for this paradoxical result. These data confirm those obtained with the antisense oligodeoxynucleotide to IGF-1 receptor RNA (Fig. 3), *i.e.*, that the activation of the IGF-1 receptor by its ligand is necessary for the growth of these cells in culture.

This is also true even when the cells are grown, not in serum-free medium, but in serum-supplemented medium. For instance, Fig. 7 shows that DU-145 cells growing in serum-supplemented medium are inhibited by JB3, which is another peptide analogue of IGF-1 (18). A scrambled peptide, JB4, which has the same amino acid composition of JB1, but in a random sequence, had no effect (data not shown).

## DISCUSSION

The 3 cell lines described in this paper are well established cell lines that have been adapted to grow *in vitro* and that originated from sources of human prostatic cancer. None of them has a normal karyotype; indeed all 3 are grossly aneuploid (20). PC-3 cells are epithelial and originated from a human prostatic adenocarcinoma metastatic to the bone (7, 21). LNC cells were also established from a metastatic lesion of human prostatic carcinoma; they are tumorigenic in nude mice and are responsive both to estrogens and to dihydrotestosterone (8). Because these cell lines are so highly undifferentiated, the conclusions that we can draw from our results are subject to reservations, no more so, though, than with any other cancer cell line in culture.

With the proper reservations, we can therefore state that these prostatic cancer cell lines are capable of growing in serum-free medium, a feature they share with other undiffentiated cell lines that have

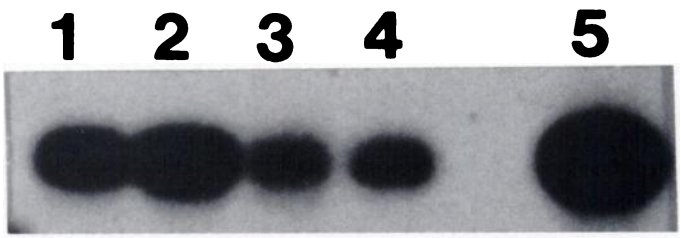


Fig. 4. Levels of IGF-1 receptor RNA in prostatic cancer cell lines. The amount of RNA was determined from cells incubated in serum-free medium for 48 h by RT-PCR. Methodologies and controls are given in "Materials and Methods." *Lane 1*, PC-3 cells; *Lane 2*, DU-145 cells; *Lane 3*, LNC cells; *Lane 4*, WI-38 human diploid fibroblasts; *Lane 5*, p12 cells, which overexpress IGF-1 receptor RNA (2).

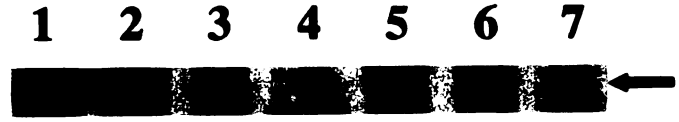


Fig. 5. Autophosphorylation of the IGF-1 receptor in prostatic cancer cell lines. IGF-1 receptor autophosphorylation was carried out as described in "Materials and Methods." Protein lysates were obtained from cells growing for 48 h in serum-free medium. Before lysis, the cells were treated for 2 h with sodium orthovanadate (1 mM). *Lane 1*, p12 cells, 48 h, in serum-free medium; *Lanes 2–4*, PC-3, DU-145, and LNC cells, respectively, 48 h in serum-free medium and 15 min after the addition of IGF-1 (3 ng/ml); *Lanes 5–7*, same as *Lanes 2–4* but without the addition of IGF-1.

Table 1 *Secretion of IGF-1 by prostatic cancer cell lines*[a]

| Cell line | IGF-1 (ng/ml/2 × $10^6$ cells) |
|---|---|
| DU-145 | 12.06 |
| LNC | 14.38 |
| PC-3 | 24.36 |

[a] The amount of IGF-1 was determined by radioimmunoassay (see "Materials and Methods") in the medium of cells incubated for 72 h in serum-free medium.

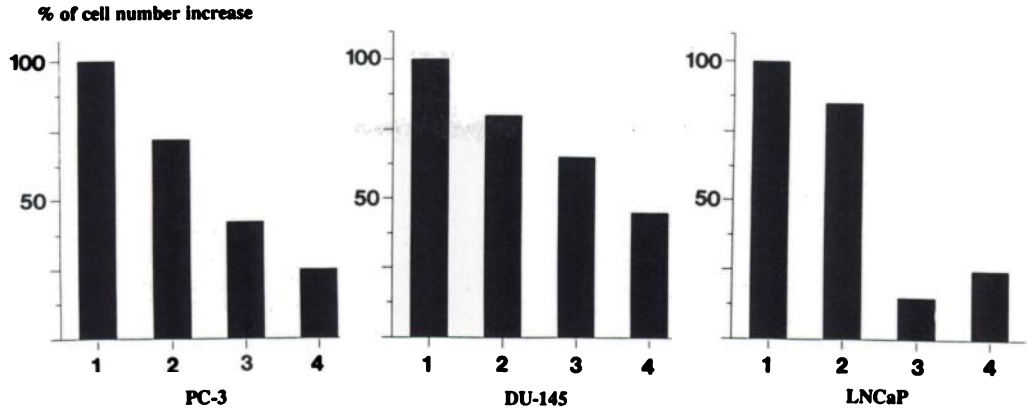


Fig. 6. Effect of IGF-1 peptide analogues on the growth of prostatic cancer cell lines. Cell lines were prepared for growth in serum-free medium as described in Figs. 1 and 2, at which time the peptide analogue JB1 was added. The cells were counted 48 h later. The results are expressed in percentage increase in the number of cells from 48 to 96 h after plating. *Bar 1*, no inhibitor; *Bar 2*, JB1, 100 ng/ml; *Bar 3*, JB1, 500 ng/ml; *Bar 4*, JB1, 1.0 μg/ml.

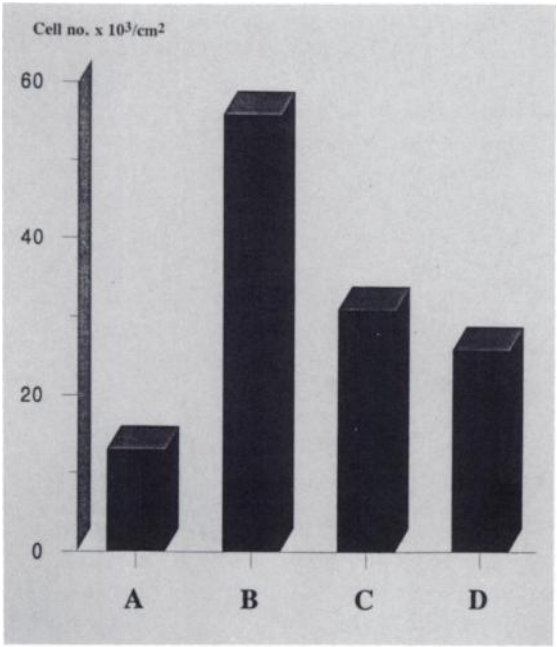


Fig. 7. Effect of an IGF-1 peptide analogue on the growth of DU-145 cells in serum-supplemented medium. The cells were plated as in Fig. 1. After 24 h, JB3 was added at concentrations of 0 (*A*), 0.1 (*B*), 0.5 (*C*), and 1.0 (*D*) μg/ml. The number of cells (*ordinate*) was determined 48 h later.

More important, the growth of the prostatic cancer cell lines is inhibited by antisense oligodeoxynucleotides to IGF-1 receptor RNA and by peptide analogues of IGF-1, indicating that these cells need a functionally activated IGF-1 receptor for growth. The specificity of the antisense oligos used in these experiments has been documented in previous articles from this laboratory (2, 4, 16) and the inhibitory effect of the IGF-1 peptide analogues on IGF-1-mediated cell growth has been detailed in the article by Pietrzkowski *et al.* (18). These analogues are particularly interesting because they are nontoxic at the concentrations used and are very effective and easy to deliver.

Their potential usefulness in the treatment of prostatic cancer and other forms of abnormal growth lies in the observation that IGF-1 is a required growth factor for a wide variety of cell types (reviewed in Ref. 1) and its action seems to be located downstream from other growth factors receptors (4, 16). In other words, while cells could circumvent other growth factor requirements by establishing an IGF-1/IGF-1 receptor autocrine loop (as in the present case), for many cell types, the activation of the IGF-1 receptor is the last receptor-mediated event before DNA synthesis and mitosis, and, presumably, cannot be circumvented except by intracellular substrates of the IGF-1 receptor. Whether this hypothesis is or not correct, the present experiment clearly indicate that the IGF-1/IGF-1 receptor pathway plays an important role in the growth of prostatic cancer cell lines and that peptide analogues of IGF-1 can effectively inhibit their growth *in vitro*.

As mentioned above, protoocogenes like c-*myb* (3) and viral DNA oncogenes like the SV40 T-antigen (5) can alter the growth factor requirements of cells by increasing the expression of IGF-1 mRNA and the secretion of IGF-1 in the medium. Recently, Isaacs *et al.* (25) reported that wild-type *p53* inhibits the growth of the same prostatic cancer cell lines described in this article. This raises the legitimate question of whether wild-type *p53* may act by inhibiting the expression of IGF-1, a hypothesis that can be easily tested.

In conclusion, our experiments show that these prostatic cancer cell lines grow by an autocrine mechanism involving the activation of the IGF-1 receptor by its ligand and that this autocrine stimulation of growth can be effectively inhibited by antisense oligodeoxynucleotides to IGF-1 receptor RNA or by analogues that compete with IGF-1 for the receptor. The possibility of practical applications should be considered.

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