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Effect of Decapeptyl® (a GnRH analogue) and of Transforming Growth Factor-α (TGF-α), in the Presence of Heparin, on the Sulfatase Activity of Human Breast Cancer Cells

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The effects of the polypeptide Decapeptyl® (a gonadotropin-releasing hormone (GnRH) agonist analogue) and of transforming growth factor-α (TGF-α), on estrone sulfate-sulfatase activities in the homogenates of various breast cancer cell lines were studied in the presence of heparin. In hormone-dependent MCF-7 breast cancer cells, Decapeptyl® can inhibit sulfatase activity, and this effect is significantly augmented in the presence of heparin. In the other hormone-dependent T-47D breast cancer cell line, the decrease of sulfatase activity was only significant when Decapeptyl® was associated with heparin. No significant effect on sulfatase activity elicited by heparin, Decapeptyl® or a mixture of both was found in the hormone-independent MDA-MB-231 breast cancer cells. TGF-α stimulates sulfatase activity in the MDA-MB-231 cells but has no effect in the MCF-7 cells; in contrast, TGF-α combined with heparin provokes a decrease of the sulfatase activity in both cell lines. It is concluded that the sulfatase activity in some types of breast cancer cell can be inhibited by heparin combined with the polypeptides Decapeptyl® or TGF-α.

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INTRODUCTION

There is substantial information whereby human mammary cancer tissues contain the enzymes necessary for the bioformation of estradiol, the hormone which plays an important role in growth and evolution of this disease [1–3]. In breast cancer tissue, estrogens can originate through two main pathways: one "via sulfatase", which transforms estrone sulfate to estrone [4–8], the other which converts androgens to estrogens "via aromatase" [9–11], and there is quantitative evidence that the sulfatase pathway is 10–100 times more important than the aromatase activity [12–14].

Previous studies in this and other laboratories have shown that different substances (e.g. Promegestone, Danazol, ICI 164,384) can inhibit the sulfatase activity in different breast cancer cell lines (e.g. MCF-7, T-47D, MDA-MB-231) [15–18]. In another series of studies using intact MCF-7 cells, we demonstrated that Decapeptyl®, a gonadotropin-releasing hormone (GnRH) agonist analogue, can also inhibit sulfatase activity and it was observed that this GnRH analogue, in the presence of heparin provokes a significant increase in sulfatase inhibition [19].

Heparin is a naturally occurring linear polyanionic sulfated glycosaminoglycan with multiple functions; for instance, complexes formed by heparin with growth factors, steroid receptors or enzymes play important biological roles [20–24].

Since growth factors (e.g. EGF, TGF-α, IGFs, FGF) can act through autocrine, paracrine or intracrine processes on breast tumor growth and can also modulate some enzymatic activities, such as 17β-hydroxysteroid dehydrogenase [25], it was interesting to investigate whether heparin alone or combined with Decapeptyl® or with the transforming growth factor-α (TGF-α) could exert a "direct effect" on the sulfatase activity in the homogenates of human breast cancer cells.

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EXPERIMENTAL

Chemicals

[6,7-^{3}H(N)]estrone sulfate (E_1S), ammonium salt (sp. act. 49 Ci/mmol) and [4-^{14}C]estrone (E_1) (sp. act. 51 mCi/mmol) were purchased from New England Nuclear Division (Du Pont de Nemours, Les Ulis, France). The purity of the radioisotopes was assessed by thin-layer chromatography in the appropriate system before use. E_1S and heparin, sodium salt (from porcine intestinal mucosa) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Decapeptyl® [D-Trp^6-gonadotropin-releasing hormone (GnRH): pyro-Glu-His-L-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly (NH_2)] was a gift from Ipsen-Biotech (Paris, France). Human recombinant transforming growth factor-α (TGF-α) was obtained from Gibco-BRL (Paisley, Scotland).

Cell culture

The hormone-dependent MCF-7 and T-47D, and the hormone-independent MDA-MB-231 human mammary cancer cell lines were kindly provided by Drs M. E. Lippman and R. B. Dickson (Georgetown University, Washington, U.S.A.). The cells were cultivated in Eagle's Minimal Essential Medium (MEM) containing 10 mM HEPES (pH 7.6), supplemented with 2 mM L-glutamine, 100 U/ml penicillin–streptomycin (A.T.G.C., Noisy-Le-Grand, France) and 5% fetal calf serum (FCS) (D.A.P., Vogelgrun, France) for T-47D and MDA-MB-231 cells, or 10% FCS for MCF-7 cells, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Media were changed twice a week. The cells were passed every 12 days and replated in 75 cm^2 flasks (A.T.G.C.) at 3×10^6 cells/flask for MCF-7 and T-47D and 1×10^6 cells/flask for the MDA-MB-231 cells. Four days prior to the experiments the cells were transferred to MEM containing 5% steroid-depleted FCS previously treated overnight at 4°C with dextran-coated charcoal (DCC) (0.1–1% w/v, DCC–FCS).

Determination of estrone sulfate-sulfatase activity

Preconfluent cells (three 75 cm^2 flasks) were washed twice with ice-cold Hank's Buffered Saline Solution (calcium–magnesium-free) and harvested by scraping. The cell solutions were pooled and centrifuged at 900 *g* for 10 min. The pellet was incubated for 10 min with cold 1.5 mM $MgCl_2$, homogenized in a teflon–glass Potter–Elvehjem homogenizer and diluted (1:1, v/v) with 0.04 M Tris–HCl buffer (pH 6.5).

The homogenate was centrifuged at 200,000 *g* for 30 min and the pellet resuspended in 0.02 M Tris–HCl buffer (pH 6.5) and sonicated to obtain the enzymatic preparation. All procedures were carried out at 0–4°C. The estrone sulfate-sulfatase activity was evaluated according to MacIndoe [26]. Briefly, enzyme assays (0.3 ml reaction volume) consisted of a 100 μl sample (0.1–0.15 mg protein) and 200 μl 0.02 M Tris–HCl buffer (pH 6.5) containing [6,7-^{3}H(N)]E_1S, ammonium salt, diluted with an excess of unlabeled substrate to obtain the required molarity (final sp. act. approx. 15,000 dpm/nmol). The protein concentrations were determined by the coomassie brilliant blue dye complexing method [27]. All assays were carried out in duplicate at 37°C in a shaking water bath. The sulfatase assay was initiated by adding the enzymatic preparation pre-incubated at 37°C for 5 min, in the presence or absence of Decapeptyl®, heparin or TGF-α. Decapeptyl® was dissolved in propane-1,2-diol. The final concentration of propane-1,2-diol in the assays was <0.1%. Blank tubes in which buffer was substituted for enzyme preparation were included in each assay. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 0.3 ml cold 0.1 M sodium bicarbonate containing 5000 dpm [4-^{14}C]E_1, to determine the recovery of the extraction process and correct the amount of tritiated product obtained. Unconjugated steroids were extracted by addition of 2 ml of toluene and centrifugation at 4000 *g* for 15 min. Following freezing of the aqueous phase, the organic phase was transferred to a liquid scintillation vial. The solvent was evaporated to dryness and 3 ml of Opti-Fluor (Packard, Rungis, France) were added. Vials were analyzed for [^{3}H] and [^{14}C] content with quench correction by external standardization. The reaction rates were linear within the incubation times and the protein range employed.

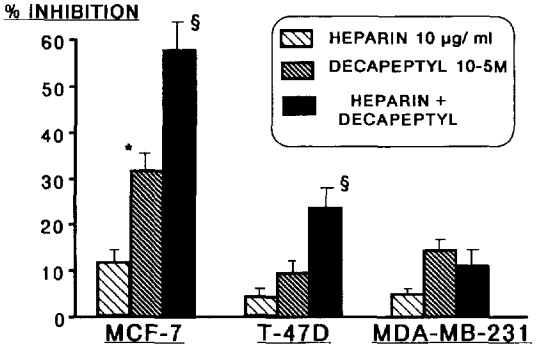

Fig. 1. Effect of Decapeptyl® and heparin, alone or combined, on the estrone sulfate (E_1S)-sulfatase activity in the homogenates of breast cancer cell lines. E_1S-sulfatase activity was determined on the homogenates of the estrogen-dependent MCF-7 and T-47D, and the estrogen-independent MDA-MB-231 breast cancer cells, as indicated in the Experimental section. The concentrations of the different compounds were: E_1S, 2 μM; heparin, 10 μg/ml; and Decapeptyl®, 10^{-5} M. Control values correspond to 485 ± 57; 2200 ± 194, and 4530 ± 720 pmol/mg protein/h for MCF-7, MDA-MB-231 and T-47D cells respectively. Percentage of inhibition was obtained by calculating the ratio (control − test)/control × 100. Data are the mean ± SEM of duplicate determinations of 3–4 experiments. *$P \leq 0.05$ vs control values. §$P \leq 0.005$ vs Decapeptyl® values.

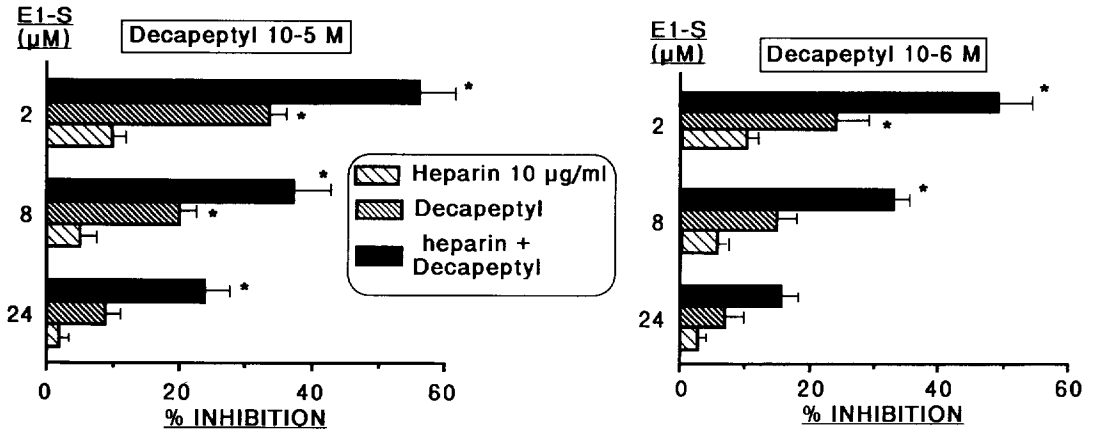

Fig. 2. Dose–response of Decapeptyl® and heparin on E_1 S-sulfatase activity in the homogenate of MCF-7 breast cancer cells. E_1 S-sulfatase activity was determined on the homogenates of the MCF-7 breast cancer cells as indicated in Experimental. The concentrations of the different compounds were: E_1 S, 2, 8, and 24 μM; heparin, 10 μg/ml; and Decapeptyl®, 10^{-5} and 10^{-6} M. Percentage of inhibition was obtained by calculating the ratio: (control − test)/control × 100. Data are the mean ± SEM of duplicate determinations of 3–4 experiments. *$P \leq 0.05$ vs control values.

Statistical analysis

Data are expressed as the mean ± SEM values. Student's t-test was used to assess the significance of the differences between means. P values ≤ 0.05 were considered significant.

RESULTS

Effect of Decapeptyl® and heparin, alone or combined, on E_1 S-sulfatase activities in the homogenates of MCF-7, T-47D and MDA-MB-231 human breast cancer cells

Figure 1 shows the comparative data of the effect of Decapeptyl® and heparin, alone or combined, on the activity of E_1 S-sulfatase in the homogenates of the breast cancer cell lines studied. Decapeptyl® alone, at 10^{-5} M, has a significant inhibitory effect (32% inhibition vs control) on the sulfatase activity of MCF-7 cells, whereas the effects are not significant in MDA-MB-231 and T-47D cells. Heparin alone (10 μg/ml) has no significant effect on the activity of this enzyme in any of these three cell lines. However, the mixture of Decapeptyl® (10^{-5} M) and heparin (10 μg/ml) provokes a potent increase of the inhibitory effect (57%) on the MCF-7 cells, and 26% in the T-47D cells. No effect is found in the MDA-MB-231 hormone-independent cells. Consequently, it is suggested that the combined effect of Decapeptyl® + heparin is a function of the cell type.

Dose effect of Decapeptyl® and heparin on E_1 S-sulfatase activity in the homogenates of MCF-7 cells

The inhibitory effect of Decapeptyl®, either alone or in association with heparin (10 μg/ml), is dependent on the concentration of estrone sulfate (in the range of 2–24 μM) and to a slighter extent on that of Decapeptyl® (10^{-5} and 10^{-6} M) as shown in Fig. 2. No significant difference in sulfatase activity was observed using heparin alone at various concentrations (Fig. 3). It is notable that the inhibitory effect of the mixture of heparin + Decapeptyl® (10^{-5} M) on the sulfatase activity is dose-dependent and increases significantly with the concentration of heparin (Fig. 3). When 1 μg/ml heparin was added to Decapeptyl® the inhibitory effect differed very little in relation to that obtained with Decapeptyl® alone, whereas the synergic effect was significant at the concentrations of 10 and 100 μg/ml heparin.

Effect of transforming growth factor-α (TGF-α) in the absence or presence of heparin on E_1 S-sulfatase activities in homogenates of MCF-7 and MDA-MB-231 cells

It is well established that growth factors can regulate the growth and the differentiation of breast tumors by

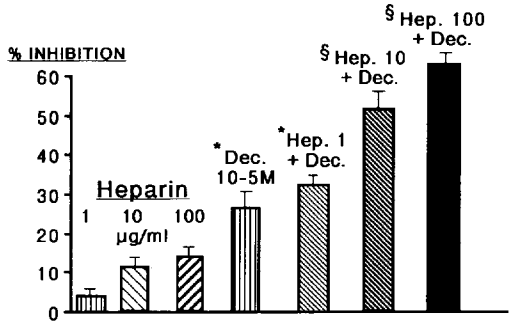

Fig. 3. Dose–response of heparin, alone or combined with Decapeptyl®, on the E_1 S-sulfatase activity in the homogenate of the MCF-7 breast cancer cell line. E_1 S-sulfatase activity was determined on the homogenates of the MCF-7 breast cancer cells as indicated in Experimental. The concentrations of the different compounds were: E_1 S, 2 μM; heparin, 1, 10 and 100 μg/ml; and Decapeptyl®, 10^{-5} M. Percentage of inhibition was obtained by calculating the ratio: (control − test)/control × 100. Data are the mean ± SEM of duplicate determinations of 3–4 experiments. *$P \leq 0.05$ vs control values. §$P \leq 0.05$ vs Decapeptyl® values.

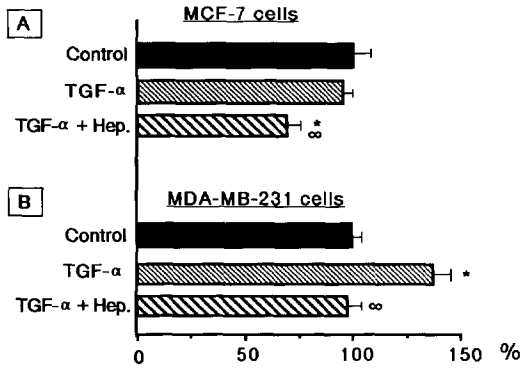

Fig. 4. Effect of transforming growth factor-α (TGF-α), alone or combined with heparin, on the E_1S-sulfatase activity in the homogenate of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells. The concentrations of the different compounds were: E_1S: 2 μM; heparin: 10 μg/ml; and TGF-α: 50 ng/ml. The mean values of the sulfatase activity (E_1S alone, control) were: 395 ± 45 and 1780 ± 132 pmol/mg protein/h for MCF-7 and MDA-MB-231 cells, respectively. These values were assigned 100%. Data are the mean ± SEM of duplicate determinations of 2–3 experiments. *$P \leqslant 0.05$ vs control values, $^{\infty}P \leqslant 0.05$ vs TGF-α values.

an autocrine, paracrine or intracrine process [28]. TGF-α belongs to one of the heparin-binding growth factor families [29]. As TGF-α is an estrogen-regulated gene secreted by hormone-dependent breast cancer cells, and is also constitutively expressed in hormone-independent cells [30, 31], we investigated whether this growth factor alone or combined with heparin could have an effect on the sulfatase activity in the homogenates of MCF-7 and MDA-MB-231 cells. TGF-α alone had no effect on the E_1S-sulfatase activity of the MCF-7 cells, but it had a significant stimulatory effect in the MDA-MB-231 cells (+36% vs control value) (Fig. 4). This effect was dose-dependent (data not shown). When heparin was associated with TGF-α, the sulfatase activity significantly decreased in both cell lines (Fig. 4).

DISCUSSION

The present data show very clearly that heparin is an inhibitory modulator of estrone sulfate-sulfatase activity in human breast cancer cells when it is associated with polypeptides, such as Decapeptyl® or transforming growth factor-α (TGF-α). As heparin alone has no significant effect, it is suggested that this inhibitory capacity could be due to the association of heparin with these polypeptides.

The effect of heparin in combination with Decapeptyl® on the homogenates of breast cancer cells (present data) agrees with results of previous studies conducted in this laboratory in which a similar effect was obtained using intact MCF-7 cells [19]. The mechanism of this effect is not clear, but as in previous studies we advanced the hypothesis that the sulfatase enzyme is localized inside the cells but in the presence of the sulfate is brought to the cell membrane where the hydrolysis takes place [8, 12, 32], we can suggest that the sulfated glycosaminoglycan heparin, associated with GnRH agonist analogue or TGF-α, can modify the activity of the enzyme.

It is well known that GnRH agonist analogues (e.g. Decapeptyl®, Buserelin, Zoladex) provoke a biochemical castration through reversible suppression of gonadotropic activity and, consequently, abolish the production of ovarian steroid hormones [33–35]. Clinical studies with GnRH agonists performed in premenopausal women with breast cancer demonstrated an effective symptom-free disease in 40% of the patients [35], and a 40% regression of the tumor volume [36]. GnRH agonists administered to postmenopausal patients can also respond to this treatment [37]. In addition, it was shown that patients with ER negative tumors can also respond to the treatment with Decapeptyl® [38].

The present data shown in Fig. 1 indicate that, when Decapeptyl® is incubated alone, or in combination with heparin, the sulfatase activity decreases significantly in the homogenate of MCF-7 cells. The potentiating effect of heparin on the inhibition of sulfatase by Decapeptyl® was also significant in the T-47D hormone-dependent cells, in which the activity of this enzyme is 8–10 times more intense than in MCF-7 cells [17]. However, in the hormone-independent MDA-MB-231 cells, Decapeptyl® alone or associated with heparin shows no significant effect. This activity difference in these two types of breast cancer cells needs to be explored.

Recent findings demonstrated that GnRH analogues can have a direct effect by inhibiting cell growth and by interacting with GnRH receptors in MCF-7 cells and breast tumors [39–41]. The evaluation of the affinity of Decapeptyl® to the receptor shows that it is higher than that of GnRH itself [42] suggesting that GnRH and its analogues can be involved in the control of the tumor growth. It is interesting to mention that GnRH analogue can inhibit the estrogen-induced increase of uterine cell proliferation [43].

It is well established that steroids, polypeptides and growth factors intimately interact to regulate the growth and differentiation of breast cancer [44]. These growth factors can modulate the activity of aromatase [45], 17β-hydroxysteroid dehydrogenase [25] or sulfatase [46]. Concerning sulfatase, Purohit *et al.* [46] have described that basic-fibroblast growth factor (b-FGF) and insulin-like growth factor-I (IGF-I) increase the activity of the enzyme in MCF-7 or MDA-MB-231 cells. It was suggested that the rate of sulfatase synthesis is implicated in this regulation, since cycloheximide abolishes the stimulatory effect provoked by these growth factors.

The control of the enzymatic activity could take place at different steps. Recently, we demonstrated that Promegestone (R-5020) not only reduces the sulfatase

activity [15, 18] but also decreases the expression of the sulfatase mRNA in breast cancer cells [47].

The present data show that TGF-α alone stimulates the sulfatase activity in MDA-MB-231, but is ineffective in MCF-7 cells; however, the combination of heparin and TGF-α decreases the sulfatase activity in both cell lines. The specificity of action of TGF-α in the hormone-independent MDA-MB-231 cells, could be related to the concept that this type of cell became more permissive due to a greater secretion of TGF-α [48, 49].

Recently, a 30 kDa TGF-α-related species has been isolated from the conditioned medium of the MDA-MB-231 cells, which has the peculiar property of binding heparin-sepharose. This purified growth factor is stimulatory on cells containing EGF-receptors, such as hormone-dependent breast cancer cells, and inhibitory on cells expressing high levels of c-*erbB*-2, such as hormone-independent cells [29, 31]. These different actions of growth factors on hormone-dependent and independent breast cancer cells were also found in previous studies of this laboratory [50]. The present findings could be related to recent studies which suggested that the heparin-binding growth factors secreted by the breast cancer cells are implicated in autocrine growth and angiogenesis of this tumor [51]. In addition, it is suggested that these heparin-binding growth factors (e.g. TGF-α, EGF, FGFs . . .) could be implicated in the evolution of hormone-dependent breast cancer cells to estrogen-autonomous growth [52–54].

As shown in this study, the activity of growth factors (such as TGF-α) can be regulated via interaction with binding molecules (such as heparin), which may either enhance or inhibit ligand activity. Another interesting example of this mechanism is the action of insulin-like growth factors I and II (IGFs) in breast cancer, which can be modulated by the presence of at least six specific binding proteins (IGFBPs). The activity of IGFs can be mediated both positively and negatively by interaction with IGFBPs. The expression of IGFBPs seems selectively different for ER positive or ER negative cell lines [55, 56].

The data indicating that heparin can decrease the effect of TGF-α and increase the inhibitory effect of Decapeptyl® on sulfatase activity could be related to the fact that the sulfated polyanionic structure of heparin can complex with different basic proteins by electrostatic bonds or by specific interactions [22]. Therefore, it has been suggested that the sequence of acidic-FGF or placental lectins corresponding to the binding of heparin strongly involves a tryptophane, histidine or arginine amino acid residues [57, 58]. It is to be remarked that this type of sequence is present in the structure of Decapeptyl®.

In conclusion, the data that Decapeptyl® associated with heparin strongly decreases the activity of estrone sulfate-sulfatase, and the finding that an association of heparin with TGF-α provokes a diminution of the sulfatase activity in breast cancer cells, opens new pathways in the knowledge of the control of sulfatase activity in mammary carcinoma. The clinical applications of these findings to the treatment of breast cancer, and the anti-tumoral effects of these associations of heparin with polypeptides, need to be explored.

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