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FROM EMERGING BIOLOGICAL INSIGHTS TO NOVEL TREATMENT STRATEGIES IN PROSTATE CANCER

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SUNTO. – Il tumore della prostata è androgeno-dipendente nelle sue fasi iniziali. Gli agonisti del GnRH, mediante la desensitizzazione degli specifici recettori ipofisarici e la conseguente soppressione della secrezione di testosterone, rappresentano la terapia di scelta per questa patologia. Dopo un periodo di remissione iniziale, il tumore progredisce verso lo stadio più aggressivo di resistenza alla castrazione (CRPC). In questa fase, il trattamento classico è rappresentato dalla chemioterapia (*i.e.*, docetaxel), che però sinora ha portato a risultati deludenti. Per questo motivo, l'identificazione di nuovi meccanismi molecolari alla base della progressione del tumore prostatico è fondamentale per lo sviluppo di nuove opzioni terapeutiche per questa patologia ad oggi incurabile. I recettori per il GnRH sono espressi in cellule di tumore prostatico CRPC e la loro attivazione ne inibisce in modo significativo la proliferazione ed il comportamento metastatico ed interferisce con il processo angiogenico. Questi risultati indicano che i recettori del GnRH espressi a livello tumorale rappresentano un valido target molecolare per lo sviluppo di nuove strategie terapeutiche per questa patologia.

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ABSTRACT. – Prostate cancer is androgen-dependent in its initial phase. GnRH agonists, through desensitization of pituitary GnRH receptors and subsequent suppression of

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testosterone secretion, represent the therapy of choice for this pathology. After an initial phase of remission, prostate cancer progresses towards its most aggressive phase of castration-resistance (CRPC). Since conventional chemotherapy treatments (*i.e.*, docetaxel) have provided scant benefit, the identification of the molecular mechanisms underlying prostate cancer progression will definitely help increase the therapeutic options for this almost incurable pathology. Receptors for GnRH agonists are expressed in CRPC cells and their activation significantly inhibits the proliferative and the metastatic behavior of cancer cells, and interferes with the angiogenic process. These data support the notion that locally expressed GnRH receptors represent an effective molecular target for novel therapeutic strategies for CRPC.

1. Introduction

Prostate cancer is the most frequent malignancy and the second leading cause of male cancer deaths in Western countries [1]. In its earlier stages, the disease is androgen-dependent; therefore, even if the majority of patients are effectively cured with definitive primary treatments, such as radical prostatectomy or radiation therapy, for high-risk locally advanced or metastatic prostate cancers the most effective treatment is represented by androgen ablation therapy, aimed at blocking androgen secretion/activity [2, 3]. This therapy includes chemical castration, which can be achieved either by gonadotropin-releasing hormone (GnRH) agonist monotherapy or by a GnRH agonist in combination with a pure antiandrogen, such as flutamide or bicalutamide (combined androgen blockade) [4-7]. Combined androgen blockade, avoiding the initial flare and providing long-term maximal androgen deprivation, demonstrated an overall survival advantage compared with GnRH agonist monotherapy [3, 5, 7, 8]. Unfortunately, after an initial phase of remission, prostate carcinoma almost inevitably progresses towards a condition of hormone-resistance (castration-resistant prostate cancer, CRPC), which is characterized by a high proliferation rate and by a strong metastatic behavior [9-11]. Conventional chemotherapy treatments (*i.e.*, docetaxel) have historically provided scant benefit for CRPC patients [12, 13]. Failure of chemotherapy and progression of prostate cancer may be caused by different molecular mechanisms, including: reactivation of the androgen receptor axis (androgen receptor mutations, amplification); multidrug resistance mechanisms, specifically by escape from apoptosis (increased ratio between anti- and proapoptotic factors); activation of growth factor sig-

naling pathways (such as EGF, IGF-I and their receptors) [14-17]. In particular, prostate cancer cells produce IGF-I, express IGF-I receptors (IGF-IR) and exhibit a significant responsiveness to the mitogenic activity of IGF-I *in vitro* [14, 18, 19]. In *in vivo* studies (performed in immunodeficient mice), the progression of androgen-dependent prostate cancer xenografts (derived from the human LAPC-9 and LNCaP cell lines) towards a phase of androgen-independence was reported to be associated with increased levels of both IGF-I and IGF-IR [20]; similarly, prostate IGF-I mRNA expression was found to increase during prostate cancer progression in TRAMP mice and to be elevated in the accompanying metastases [21]. In men, high serum levels of IGF-I have been reported to be associated with an increased risk of prostate cancer [22]. Interestingly, IGF-I has been reported to increase both the invasive and the migratory behavior of CRPC cells [23, 24], indicating that this growth factor might regulate not only the growth but also the progression of prostate cancer towards its highly metastatic phase.

The clarification of the molecular mechanisms of castration-resistant prostate cancer development will help increase the therapeutic options for this almost incurable pathology.

Gonadotropin-releasing hormone (GnRH) was first identified as the hypothalamic key regulator of the reproductive functions. By binding to specific receptors (GnRH-R) on pituitary gonadotropes, this decapeptide activates the pituitary-gonadal axis [25]. Synthetic superactive agonists of GnRH, when given continuously and at high doses, suppress gonadal steroid secretion through the desensitization of pituitary GnRH-R. On the basis of their activity, these compounds represent the most widely and successfully utilized medical treatment for androgen-dependent prostate cancer [5, 26].

Later, it was shown that GnRH-R are also expressed in cancer tissues where they act as negative regulators of tumor growth [27-34]. In particular, Limonta's laboratory has previously reported that GnRH-R are expressed in prostate cancer cells, specifically in CRPC cells; activation of these receptors by means of GnRH agonists significantly reduces the proliferation of prostate cancer cells, *in vitro* [35-37] and *in vivo*, when xenografted into immunodeficient nude mice [38]. In prostate cancer cells, GnRH-R are coupled to the pertussis toxin-sensitive G_{alphai} protein and their activation interferes with growth factor-induced mitogenic signals [19, 39]. These data indicate that GnRH

agonists exert a direct and significant antiproliferative effect on castration-resistant prostate cancer cells. To this purpose, it must be underlined that GnRH receptors are expressed also in prostate cancer specimens and their expression persists at relapse after hormone ablation [40]. A better understanding of the molecular mechanisms of the antitumor activity of these compounds will definitely lead to the identification of tumor GnRH-R as a molecular target for the development of novel receptor-targeted therapeutic strategies for this pathology.

Based on these observations, the experiments here described were performed to investigate whether GnRH agonists (Leuprolide) might affect the migratory/invasive/angiogenic properties of CRPC, by interfering with the activity of growth factors, such as IGF-I and VEGF. A possible link between GnRH-R activation and the apoptotic machinery were also investigated.

2. Results and Discussion

2.1 GnRH agonists inhibit the migratory/invasive behavior of castration-resistant prostate cancer cells by interfering with the expression/activity of IGF-I

Preliminary experiments were performed to confirm the antiproliferative effect of Leuprolide on castration-resistant DU145 prostate cancer cells, as previously reported [37]. Then, the effect of Leuprolide on the ability of prostate cancer cells to migrate towards an extracellular matrix ligand (vitronectin) was investigated by means of a haptotactic assay. It was observed that, when DU145 cells (grown in serum-supplemented medium) were pretreated with Leuprolide for 4 days before being seeded in a vitronectin-coated Boyden's chamber, the number of the cells that migrated towards vitronectin was significantly decreased when compared to control cells.

Experiments were then performed to verify whether Leuprolide might affect the invasive behavior of DU145 prostate cancer cells. The effects of Leuprolide were evaluated on the ability of DU145 cells to invade a matrix of reconstituted basement membrane (Matrigel). DU145 cells form aggregates in Matrigel, when prepared by the hanging-drop technique, and spontaneously leave the aggregates and invade the Matrigel preparation at 4 and 9 days, when grown in serum-sup-

plemented medium. Treatment of the cells with Leuprolide completely abrogated their migration at the two time intervals considered.

Limonta's laboratory has previously shown that, in DU145 prostate cancer cells, GnRH agonists (Zoladex) reduce the concentration of IGF-IR, as measured by radioreceptor assay, as well as the IGF-I-induced phosphorylation of the IGF-IR [19]. Experiments were then performed to clarify whether similar effects might be elicited by the GnRH agonist Leuprolide. By Western blot analysis, it could be demonstrated that treatment of DU145 cells with Leuprolide substantially reduced the expression of the IGF-IR protein. To evaluate whether Leuprolide might interfere also with the phosphorylation of the IGF-IR, DU145 cells were treated with the GnRH agonist for either 1 or 2 h before being exposed to a 3-minutes stimulus with IGF-I. It was found that the IGF-I receptor is not phosphorylated in DU145 cells cultured in the absence of serum, indicating that, in basal conditions, the autocrine IGF-I is not sufficient to activate its own receptors. As expected, IGF-I induced the phosphorylation of IGF-IR. Leuprolide, when given alone, was completely devoid of effect, a result which was expected since in basal conditions IGF-IR was not activated. The pretreatment of DU145 cells with Leuprolide for 1 or 2 h substantially counteracted the IGF-I-dependent phosphorylation of IGF-IR. These results confirmed that Leuprolide interferes with the IGF-I system (receptor levels and phosphorylation), as previously described for the other GnRH agonist Zoladex [19].

It was previously reported that, in DU145 cells, IGF-I exerts its pro-migratory activity through the PI3-K/Akt signaling pathway [24]. Experiments were performed to verify whether Leuprolide might interfere with the phosphorylation of the Akt kinase induced by IGF-I. DU145 cells, in serum-free medium, were treated with Leuprolide for 1 or 2 h, either in the absence or in the presence of IGF-I. The level of Akt phosphorylation was determined by Western blot analysis using a phospho-Akt specific antibody. The results obtained showed that, in basal conditions, DU145 cells express undetectable levels of phosphorylated Akt; this confirms that, in the absence of serum, autocrine IGF-I is not sufficient to activate its receptor and the downstream signaling pathway. IGF-I induced a significant increase of phosphorylation of the protein, while Leuprolide was completely devoid of effect when given alone. Pretreatment of the cells with the GnRH agonist substantially counteracted the IGF-I-dependent phosphorylation of Akt at both

time intervals considered. These effects were specific since the level of expression of the unphosphorylated protein was found to be constant throughout the experiments.

In Limonta's laboratory it has been shown that IGF-I stimulates the migration of DU145 cells, and that this effect is accompanied by a rearrangement of the cytoskeleton components as well as by a change in cell morphology (as revealed by scanning electron microscopy) and in integrin expression [24].

Experiments were then performed to clarify whether Leuprolide might decrease the migratory properties of prostate cancer cells by interfering with the activity of IGF-I. DU145 cells were treated with Leuprolide for 4 days, either alone or in the presence of IGF-I during the last 24 h of treatment. Cells were then seeded in the upper compartment of a Boyden's chamber for the haptotactic assay. As expected, IGF-I significantly promoted the migration of DU145 cells (in serum-free conditions) towards vitronectin. Leuprolide, when given alone, did not affect cell migration; on the other hand, the GnRH agonist significantly prevented the pro-migratory effect of IGF-I. Similar results were obtained with another androgen-independent prostate cancer cell line (PC3). Grzmil and coworkers [23] reported that IGF-I increases the invasive behavior of androgen-independent PC3 prostate cancer cells. Experiments were performed to verify whether Leuprolide might counteract the pro-invasive activity of IGF-I on DU145 cells. Using the Matrigel invasion chamber, it was found that IGF-I significantly increased the ability of the cells to invade the Matrigel preparation and to migrate towards the lower surface of the inserts. Leuprolide, given alone, had no effect; however, the GnRH agonist completely counteracted the pro-invasive activity of IGF-I.

The metastatic behavior of tumor cells is usually associated with changes in cell morphology, rearrangements of cytoskeletal proteins, as well as alterations of the expression of cell adhesion molecules, such as integrins. Scanning electron microscopy was performed on DU145 cells treated with IGF-I and Leuprolide, either alone or in combination. It was observed that lamellipodia are not present in control cells; moreover, the bodies of IGF-I-treated cells did not adhere to the substratum. Leuprolide, alone, did not modify cell morphology; however, the GnRH agonist substantially antagonized the morphological changes induced by IGF-I.

Immunofluorescence staining was then performed to clarify

whether Leuprolide might affect the IGF-I-induced rearrangement of cytoskeletal components (actin and tubulin) in prostate cancer cells. It was found that, in DU145 cells, IGF-I induced a change of cell morphology, with acquisition of lamellipodia-like protrusions and a redistribution of the actin cytoskeleton as shown by an increased intensity of the fluorescence in peripheral areas and at the leading edge of the protrusions. Leuprolide, when given alone, did not exert any effect. DU145 cells treated with the GnRH agonist exhibited indeed a morphology and an actin cytoskeletal organization similar to that of control cells. Neither IGF-I nor Leuprolide, given alone or in combination, modified immunofluorescence staining for tubulin in DU145 cells.

It was recently reported that, in DU145 cells, IGF-I increases the expression of alphavbeta3 integrin as evaluated both by immunofluorescence and by biotinylation/immunoprecipitation followed by Western blot analysis [24]. Here, it was studied whether Leuprolide might counteract the action of IGF-I. First of all, in IGF-I-treated cells the staining of alphavbeta3 fluorescence was increased *vs.* controls, with a clear localization of the integrin at the cell membrane. Leuprolide, given alone, did not affect either the intensity of the fluorescence or the cellular localization of alphavbeta3. On the other hand, the effect of IGF-I was clearly abolished by the co-treatment of the cells with the GnRH agonist. These observations were further confirmed at the protein level. By biotinylation, immunoprecipitation and Western blot it was shown that, as expected, alphavbeta3 protein levels increased after IGF-I treatment. Leuprolide, given alone, did not affect integrin expression; however, the GnRH agonist substantially antagonized the effect of the growth factor.

Taken together, these observations strongly support the conclusion that, in castration-resistant prostate cancer cells, activation of locally expressed GnRH receptors by means of GnRH agonists exerts a significant anti-migratory and anti-invasive effect, by interfering with the expression/activity of growth factor (IGF-I) receptors. In line with these data, Gnanapragasam and coworkers [41] have shown that the GnRH agonist Buserelin substantially counteracts the pro-invasive effect of fibroblast growth factor (FGF) on DU145 cells. Moreover, Dondi *et al.* [42] have reported that Leuprolide exerts an anti-metastatic activity on castration-resistant prostate cancer cells, through the inhibition of the urokinase Plasminogen Activator (uPA) system. An antimetastatic activity for GnRH agonists has been previously reported

also for cancers not classically related to the reproductive system, such as melanoma [43] and epidermoid carcinoma [44], expressing the GnRH receptor.

The data here reported, together with our previous observations [19], indicate that, in castration-resistant prostate cancer cells, GnRH agonists might exert a direct antitumor effect (by reducing both the proliferation and the metastatic behavior of the cells), by interfering with growth factor activity.

As previously mentioned, GnRH agonists are widely and successfully used for the treatment of androgen-dependent prostate cancer, on the basis of their ability to induce chemical castration [5]. Data from Limonta's laboratory strongly suggest that locally expressed GnRH receptors might represent a molecular target for the development of novel GnRH-R-targeted therapeutic strategies also for the most advanced, and almost incurable, hormone-resistant prostate carcinoma (*Fig. 1*).

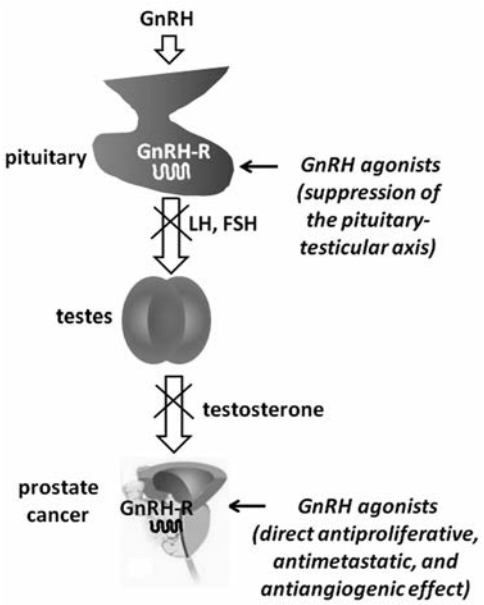

Fig. 1 – GnRH agonists, through activation of pituitary GnRH receptors, suppress testosterone secretion in androgen-dependent prostate cancer. GnRH agonists, through activation of GnRH receptors expressed at tumor level, exert a direct and significant antiproliferative, antimetastatic, and antiangiogenic effect in castration-resistant prostate cancer

2.2 GnRH agonists inhibit the angiogenic properties of human endothelial cells by interfering with the activity of VEGF

The process of angiogenesis, by inducing formation of a new vasculature, is known to play a crucial role in cancer progression and metastasis formation. New blood vessels provide the adequate supply of oxygen and nutrients to cancer cells and allow their dissemination to distant organs [45, 46]. Aim of these studies was to investigate whether GnRH agonists might affect the angiogenic process in prostate cancer.

To study whether GnRH agonists could directly affect the angiogenic properties of endothelial cells, the expression of GnRH-R was investigated in human umbilical vein endothelial (HUVEC) cells. By RT-PCR, we found that the mRNA coding for this receptor is expressed in HUVEC cells. This suggests that GnRH agonists might exert an additional, direct effect on tumor angiogenesis.

To investigate whether GnRH agonists might affect endothelial cell proliferation, HUVEC cells were treated, for 4 days, with $VEGF_{165}$, either alone or in the presence of the GnRH agonist Zoladex. As expected, $VEGF_{165}$ significantly stimulated HUVEC cell proliferation, while Zoladex, given alone, did not affect endothelial cell growth. In contrast, the GnRH agonist significantly counteracted the proliferative activity of $VEGF_{165}$. The activity of the agonist was found to be specific since it was completely counteracted by the co-treatment of the cells with a GnRH antagonist (Antide) [47].

Having established that GnRH agonists counteract VEGF-induced HUVEC cell proliferation, we then examined its implication in the angiogenic process *in vitro*. For these studies, we utilized a conventional angiogenesis test, based on the ability of endothelial cells to spontaneously form capillary-like structures, when incubated on an extracellular basement membrane matrix preparation (Matrigel). It was found that, in the absence of serum, HUVEC cells lack the ability to undergo alignement into capillary-like structures. VEGF strongly induced the process of tube formation while the GnRH agonist, given alone, was not able to affect the formation of capillaries. Zoladex substantially counteracted the effects of VEGF; the activity of this compound was shown to be specific since it was completely counteracted by the co-treatment of the cells with the GnRH antagonist Antide. Tube formation was quantified by counting the number of endothelial cell structures [47]. These results demonstrate that the activation of locally expressed

GnRH-R interferes with the VEGF-induced tubulogenic capability of endothelial cells. Thus, in prostate cancer, activation of locally expressed GnRH receptors inhibits the proliferative/metastatic as well as the angiogenic processes in prostate cancer progression (*Fig. 1*).

2.3 GnRH agonists inhibit the proliferation of castration-resistant prostate cancer cells without inducing apoptosis

Based on the strong antiproliferative effect of GnRH agonists on castration-resistant prostate cancer cells, it was hypothesized that these compounds might reduce cell number by triggering cell death, through induction of apoptotic mechanisms. Preliminary experiments, performed by fluorescence activate cell sorting (FACS) analysis, demonstrated that activation of GnRH receptors does not induce apoptosis in hormone-insensitive DU145 cells.

In agreement with these data, Morgan and coworkers [48] recently reported that GnRH agonists inhibit the growth of prostate cancer cells engineered to overexpress the GnRH receptor, by affecting the distribution of the cells throughout the cell cycle (*i.e.*, by increasing the number of the cells in the G2 phase of the cycle), without inducing cell death. Moreover, the expression of apoptosis-related genes was found to be modified by a GnRH agonist without induction of apoptosis [49].

On the contrary, GnRH agonists were described to induce cell death in androgen-independent prostate cancer cells [50] and in primary cultures of human prostate cancer cells [51, 52]. Further experiments are required to clarify the reasons for these discrepancies. In Limonta's laboratory it has hypothesized that GnRH agonists might not be responsible for apoptosis induction, but they might sensitize prostate cancer cells to the apoptotic activity of chemotherapeutic drugs (*i.e.*, docetaxel). Experiments are in progress to verify this hypothesis.

In conclusion, the studies here summarized demonstrate that GnRH receptors are expressed in CRPC cells and they are endowed with a strong antitumor activity (*Fig. 1*). GnRH agonists, through activation of these receptors, might represent a novel effective targeted strategy for the treatment of prostate cancer in its most aggressive, and almost incurable, phase of castration resistance.

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