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Summary
Evidence indicates that androgen-sensitive prostate cancer cells have a lower malignant potential. We previously demonstrated that expression of androgen receptor (AR) by transfection of the androgen-independent prostate cancer cell line PC3 decreases invasion and adhesion of these cells through modulation of α6β4 expression. Treatment with the androgen further reduced adhesion and invasion of the cells without, however, modifying α6β4. Here we investigated whether the androgen has a direct effect on α6β4-EGF receptor (EGFR) interaction and signalling leading to invasion of these cells. Immunofluorescence microscopy demonstrated that in control cells (PC3-Neo), α6β4 and EGFR colocalize and redistribute in response to epidermal growth factor (EGF). In PC3-AR cells colocalization and redistribution between the two molecules was reduced and abolished by pre-treatment with R1881. Co-immunoprecipitation studies demonstrated that tyrosine phosphorylation of β4 in response to EGF was reduced in PC3-AR cells compared to PC3-Neo. Immunofluorescence and co-immunoprecipitation studies demonstrated colocalization at membrane level and co-immunoprecipitation of EGFR and AR, indicating an interaction between the two proteins. PI3K activity, a key signalling pathway for invasion of these cells, was decreased in PC3-AR cells in response to EGF and further reduced by treatment with R1881. EGFR internalization was strongly reduced in PC3-AR compared with PC3-Neo cells and was reduced by treatment with R1881. In conclusion, the expression of AR by transfection in PC3 cells confers a less malignant phenotype by interfering with EGFR–α6β4 interaction and signalling leading to invasion through a mechanism involving an interaction between the classic AR and EGFR.

Keywords: epidermal growth factor, integrin, prostate cancer

Introduction
Prostate cancer (PC) is one of the most common cancers and the second leading cause of death among other populations than American men only (Amanatullah et al., 2000). As PC cell growth is enhanced by androgens, in the advanced stages of the disease androgen ablation therapy represents a valuable tool for the treatment of these patients. However, the development in most patients after few years of treatment of androgen-independent clones characterized by higher invasiveness and metastatic properties, has focused attention on the molecular mechanisms that lead to loss of androgen dependence as well as...
on the pathways that are regulated by androgens in these cells. At this stage no valuable tools for the treatment of the patients are available. Studies performed with patient specimens have shown that the androgen receptor (AR) is expressed in almost all cancers of the prostate after androgen ablation therapy indicating that loss of androgen–dependence is not related to the expression of AR (Van der Kwast et al., 1993). Indeed, maintenance of androgen-sensitivity appears to have a more differentiated and less malignant phenotype of PC cells. The ability to produce tumours in nude mice, for instance, is higher in androgen-insensitive cell lines (such as PC3 and DU145) with respect to the androgen-sensitive (LNCaP) (Witkowski et al., 1993). Moreover, invasion ability of androgen-independent prostate carcinoma cells in response to epidermal growth factor (EGF) is decreased by transfection with an AR expression vector (Bonaccorsi et al., 2000; Cinar et al., 2001). In this light, the role of androgens and AR in the regulation of the signalling pathways involved in invasion and metastasis represents a primary task in studies on PC biology. As a result, some androgen-regulated genes involved in signalling pathways leading to invasion has been recently identified and their role in lower invasion ability of androgen-sensitive PC cell lines indicated (Bonaccorsi et al., 2000; Sumitomo et al., 2000; Manos et al., 2001; Trusolino et al., 2001), suggesting that loss of androgen regulation leads to increased invasion through multiple pathways.

Multiple pathways resulting from extracellular and intracellular signals regulate invasion of carcinoma cells. Indeed, cell migration results from merging of signalling pathways, which employ growth factors and their receptors, adhesion receptors (integrins) and cytoskeletal elements.

Integrins are heterodimers composed of α and β subunits (Meredith et al., 1996), and have a key role in the regulation of proliferation and invasion of carcinomas (Juliano & Verner, 1993). In particular, αβ4, one of the main receptors of laminins, is a primary actor in the process of migration and invasion (Dans et al., 2001). In normal epithelia αβ4 mediates the formation of stable adhesive structures termed hemidesmosomes, that link intermediate filaments to extracellular matrix, whereas in carcinoma cells, it promotes migration on laminin through association with actin cytoskeleton (Dans et al., 2001). It has been demonstrated that αβ4 co-immunoprecipitates with ErbB-2 and EGF receptor (EGFR) to promote cell migration and invasion in response to EGF (Gambaletta et al., 2000; Dans et al., 2001). EGF stimulates phosphorylation and activation of β4 cytoplasmic domain, which leads to phosphoinositide-3 kinase (PI3K) activation and migration (Gambaletta et al., 2000; L. Bonaccorsi et al., unpublished data). In addition, it has been recently shown that αβ4 may also represent a functional amplifier of biochemical signals leading to invasion by potentiating growth factor activation of Ras- and PI3K-dependent pathways (Trusolino et al., 2001).

In a previous study we demonstrated that the expression of αβ4 is higher in androgen-independent prostate carcinoma cell lines (PC3, DU145) with respect to androgen-sensitive PC cells, LNCaP and PC3 transfected with a full length hAR expression vector (PC3-AR) (Bonaccorsi et al., 2000). PC3-AR cells were characterized by lower binding to laminin-1, anchorage-independent growth and EGF-mediated invasion compared with androgen-independent cells (Bonaccorsi et al., 2000). The treatment with the synthetic androgen R1881 determined a further decrease of the invasion ability of these cells, without however, modifying the surface expression of αβ4 (Bonaccorsi et al., 2000) and prospecting an effect of the androgen on EGF-mediated signalling related to invasion. In agreement with our results, in a different cell model, Cinar et al. (2001) have recently demonstrated, that invasion of PC cells is decreased by transfection with AR and by treatment with androgens. In order to study if androgens and AR have a direct effect on EGFR–αβ4 interaction and signalling leading to invasion in response to EGF, we investigated the effect of androgens on EGFR–αβ4 interaction, association of AR with EGFR and PI3K activation leading to invasion in PC3-AR cells in response to EGF (L. Bonaccorsi et al., unpublished data).

**Results**

**EGFR and αβ4 colocalization in response to EGF is disrupted in PC3-AR cells**

As mentioned above the interaction between EGFR and αβ4 is essential for carcinoma cell invasion (Mainiero et al., 1996; Gambaletta et al., 2000). By laser confocal immunofluorescence studies, we demonstrated that α6 and β4 colocalized with the EGFR in basal conditions in PC3-Neo cells (transfected only with the vector) on laminin-1. In response to EGF α6, β4 and EGFR redistributed and colocalized in discrete areas at the level of membrane ruffles. This pattern of colocalization of αβ4 and EGFR in response to EGF was not substantially affected by pre-treatment with androgens. On the contrary, in PC3-AR cells colocalization between α6, β4 and EGFR was reduced with respect to PC3-Neo both in basal conditions and in response to EGF. In addition, pre-treatment with androgens further reduced redistribution of αβ4 and EGFR suggesting an interference of AR and androgens with the mechanisms leading to interaction between the two proteins. Immunoprecipitation studies conducted on PC3-Neo and PC3-AR cells confirmed that this colocalization was caused by an interaction between αβ4 and EGFR. After immunoprecipitation of EGFR, β4 was detected in both cell lines and after immunoprecipitation of β4, EGFR was detected in both cell lines indicating that the two proteins are constantly associated in our cell models. By probing the blot with an
antibody against phosphotyrosine residues we detected a tyrosine phosphorylated band at 200 kDa exactly migrating as β4 and a phosphorylated band at 180 kDa corresponding to EGFR. Tyrosine phosphorylation of both bands increased in response to EGF in both cell lines, although EGF-induced tyrosine phosphorylation of β4 was much less evident in PC3-AR cells. Moreover, in PC3-AR cells autophosphorylation of EGFR in response to EGF was lower compared with PC3-Neo suggesting a reduced activation of EGFR and β4 integrin subunit by EGF in the presence of the AR.

The androgen receptor colocalizes with EGFR at the plasma membrane

Recent data indicate that besides its classical localization at the nuclear level, the AR may be targeted at the membrane level where interactions with molecules involved in growth factor signalling, such as src kinase family members (Migliaccio et al., 2000), caveolin-1 (Lu et al., 2001) and PI3K (Simoncini et al., 2000) have been demonstrated. We investigated the localization of AR and EGFR by laser confocal immunofluorescence experiments. The AR localizes both to the nucleus and the cytoplasm of PC3-AR cells, although after stimulation with androgens, increased location to the nuclei was evident. Interestingly, a marked colocalization of AR with EGFR at the plasma membrane level was present. In response to EGF, colocalization between AR and EGFR was also evident at the level of intracellular granules, reflecting internalization of the EGFR–AR complex. When PC3-AR cells were stimulated with EGF in the presence of androgens, the pattern of colocalization between the two proteins was different compared with EGF alone because no discrete granules were present. After immunoprecipitation with an antibody against EGFR, a band at 110 kDa corresponding to AR was detected in PC3-AR cells. Tyrosine phosphorylated bands corresponding to EGFR and β4 were also detected. Similarly, following immunoprecipitation of AR in PC3-AR cells, a tyrosine phosphorylated band corresponding to EGFR was also detected.

EGFR and PI-3 kinase are essential for EGF-mediated invasion in PC3-AR

An association between the EGFR and α6β4 promotes the activation of several signalling pathways including PI3K (Shaw, 2001), which finally leads to cell migration and invasion. To evaluate whether PI3K activation is involved in Matrigel invasion of PC3-Neo cells, invasion assays were performed in the absence or presence of the PI3K inhibitor LY294002. Matrigel invasion in response to EGF was inhibited by the treatment with LY294002. EGF-mediated Matrigel invasion was also suppressed by ZD1839 a tyrosine kinase inhibitor selective for EGFR (Moasser et al., 2001). To investigate whether PI3K activity was altered in PC3-AR cells, a PI3K activity assay was performed in both cell lines. We observed that basal and EGF-stimulated PI3K activity was reduced in PC3-AR cells with respect to PC3-Neo. Pre-treatment with androgens determined a further decrease of EGF-stimulated PI3K activity in PC3-AR cells, whereas it was ineffective in PC3-Neo cells.

EGFR internalization is reduced in PC3-AR with respect to PC3-Neo

Recent evidences indicate that a correct endocytotic pathway is important for EGFR signalling by controlling the specificity of the response to the agonist (Wiley & Burke, 2001). It has also been demonstrated that internalized EGFR is enzymatically active, is still phosphorylated and maintains association with many adaptor proteins (Burke et al., 2001). To investigate whether EGFR internalization following addition of the growth factor is affected by androgens, the expression of EGFR on cell surface by flow cytometry was studied. After EGF treatment (30 min), a significant decrease in cell surface expression of the receptor was detected in PC3-Neo cells. In PC3-AR cells this decrease of EGFR expression was less marked indicating a decreased internalization. Treatment with androgens further reduced EGFR internalization in response to EGF in PC3-AR but did not affect EGFR internalization in PC3-Neo cells.

Discussion

Androgen-insensitive PC cells are characterized by increased growth, adhesion, invasion and migration (Witkowski et al., 1993; Bonaccorsi et al., 2000; Cinar et al., 2001). We show here that an interaction between AR and EGFR occurs in androgen-sensitive PC cell lines and provide evidence that the reduced invasive properties of these cells are because of a disruption of the interaction between the EGFR and α6β4, decreased signalling leading to β4 phosphorylation and lower PI3K activation in response to EGF. The mechanisms of AR–EGFR interaction as well as of reduced function of EGFR in PC3-AR cells are not clear at the moment but it is possible that AR–EGFR interaction may lead to a sequestration of the EGFR. In fact our results on EGFR internalization pattern indicate a disruption of the endocytotic process in PC3-AR cells compared with parental cells. Recent data have demonstrated that AR is able to interact with plasma membrane-associated proteins such as src kinase family members (Migliaccio et al., 2000) and caveolin-1 (Lu et al., 2001) that is also involved in EGFR signalling by inhibiting EGFR tyrosine kinase activity. In agreement with these results caveolin-1 might interact with AR and EGFR mediating a molecular complex that might negatively regulate invasion by inhibiting EGFR redistribution, signalling and internalization (see Fig. 1: working hypothesis). In conclusion, in androgen-sensitive prostate carcinoma cells, androgens and AR contribute to confer a less malignant phenotype of these cells both by reducing the expression of α6β4 (Bonaccorsi et al., 2000) and by interfering with EGFR–α6β4 interaction.
and signalling leading to invasion through an interaction between AR and EGFR (L. Bonaccorsi, V. Carloni, M. Muratori, L. Formigli, S. Zecchi, C. Forti & E. Baldi, unpublished data).

References


Shaw, L. M. (2001) Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the α6β4 integrin-dependent activation of phosphoinositide-3-OH kinase and promotion of invasion, Molecular Cell Biology 15, 5082–5093.


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