Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice

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Abstract

The activation of epidermal growth factor receptor (EGF-R) plays a key role in the promotion of proliferation and invasion in prostatic carcinoma (PCa). Gefitinib (Iressa; ZD1839), an orally active EGF-R tyrosine kinase inhibitor, has shown an important anti-proliferative activity in tumors expressing EGF-R both *in vitro* and *in vivo*. Our aim was to elucidate the role of gefitinib in the modulation of the metastatic spread of PCa cells. The therapeutic role of gefitinib was investigated by evaluating the proliferative and invasive ability of the PCa cell line PC3 and of its high metastatic sub-line, PCb2, by *in vitro* assays and intracardiac injection in nude mice. The inhibitory effect of gefitinib was tested *in vivo* by injecting PCa cells subcutaneously or in the left ventricle of nude mice and by administrating daily 150 mg/kg of gefitinib. While xenograft growth was equally reduced in all PCa lines (about 50%), the bone metastasis formation was inhibited especially for the high metastatic PCb2 sub-line (81%) in comparison to PC3 cells (47%). The comparative *in vitro* analysis among PCa cell lines showed that PCb2 cells were more sensitive to the inhibitory effect of gefitinib in their invasive ability compared to parental PC3 cells but not in their proliferation rate. Moreover, PCb2 cells demonstrated an increased invasive ability *in vitro* in response to bone stromal cell conditioned medium (BCM). The simultaneous presence of 0.1 ng/ml gefitinib was sufficient to reduce the number of invaded cells in the presence of both EGF and BCM. The molecular characterization of the highly aggressive PCa sub-lines demonstrated that this phenomenon was associated with an increment in uPA/uPAR axis but not in EGF-R expression. In conclusion, our data suggest that the use of gefitinib as a therapeutic agent may be indicated in the control of PCa spreading to bone.

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Introduction

EGF-R is a 170 kDa type I transmembrane glycoprotein containing a ligand-binding ectodomain, a single hydrophobic transmembrane region, and a cytoplasmic tail which includes a tyrosine kinase domain and docking sites for a variety of signaling effectors (Jorissen *et al.* 2003). The downstream signaling network generated by EGF-R activation is able to control cell growth (Zyzak *et al.* 1994), survival (Moro *et al.* 1998) and migration (Frey *et al.* 2004). Early studies on the of EGF-R in breast and

prostate tumor cells have indicated a significant association between EGF-R activation and the acquisition of the invasive phenotype (Zolfaghari & Djakiew 1996, Kondapaka *et al.* 1997). In recent years, an increasing number of studies performed with several experimental models have reinforced this hypothesis suggesting a leading role of EGF-R during migration induced by bombesin (Madarama *et al.* 2003), invasion modulated by urokinase plasminogen activator-receptor (uPA-R) (Mamoune *et al.* 2004) and bone metastatization (Kim *et al.* 2003).

The expression level of uPA-R on disseminated tumor cells was significantly correlated with increasing tumor cell counts in bone marrow and clinical prognosis (Heiss *et al.* 1995). In particular urokinase plasminogen activator and uPA-R may contribute to the development of an active crosstalk between tumor cells and host cells. This aspect has suggested the hypothesis of a vicious circle, according to which the establishment and growth of metastatic PCa in bone is determined by an overlapping cellular loop between tumor cells and bone cells (Guisse & Mundy 1998). uPA-R is the best known modulator of uPA activation and it is expressed both by invasive PCa cells and normal osteoblasts (Rabbani *et al.* 1994). The basic importance of the uPA/uPA-R system in the determination of PCa bone metastases has been clearly demonstrated in experimental animal models by the use uPA and uPA-R inhibitors (Rabbani *et al.* 1995, Margheri *et al.* 2005). Recently, some authors have suggested an important correlation between the function of the uPA/uPA-R system and EGF-R activation. For example, EGF-R inhibitors are able to block the signal transduction generated by uPA (Jo *et al.* 2003), and EGF-R activation is required for important cellular responses stimulated by uPA, such as invasive behavior (Guerrero *et al.* 2004).

Prostate carcinoma (PCa) develops as a hormone-dependent disease. When surgical eradication of the tumor fails, because of the presence of disseminated micrometastases, androgen ablation therapy becomes the treatment of choice. However, the androgen blockade therapy is followed in the majority of patients by the development of a hormone refractory disease, dramatically lowering the median survival expectancy (Small & Vogelzang 1997). The molecular mechanisms underlying this phenomenon are scarcely known. However, experimental evidence has been provided emphasizing the role of mitogenic signaling pathways other than the androgen receptor axis (Shi *et al.* 2001). In particular, a significant correlation between the grade of PCa progression and the expression of EGF-R has been demonstrated (Di Lorenzo *et al.* 2002). In addition, IHC and microarray analyses have recently indicated that EGF-R is frequently over-expressed in hormone refractory and metastatic PCa (Hernes *et al.* 2004, Zellweger *et al.* 2005).

Gefitinib (Iressa; ZD1839), a tyrosine kinase inhibitor specific for EGF-R, has been approved as a single drug therapy for lung cancer following very encouraging data obtained in clinical trials (Cohen *et al.* 2004). To date, a substantial body of information is available in different cancer cells and xenografts describing the inhibition of cell growth by gefitinib

(Wakeling *et al.* 2002). We have previously shown that the use of gefitinib is effective in inhibiting EGF-dependent growth in PCa primary cultures and cell lines, independently of their sensitivity to androgen (Vicentini *et al.* 2003). Moreover, we and other authors have demonstrated a co-operative effect of anti-androgens and gefitinib, both *in vitro* (Festuccia *et al.* 2005b) and *in vivo* (Sirotnak *et al.* 2002). A molecular interaction between AR and EGF-R signaling has been recently described, suggesting the existence of a close link between the two pathways which control PCa progression (Bonaccorsi *et al.* 2004).

The purpose of our study was to determine whether the inhibition of EGF-R signaling pathway could attenuate the metastatic potential of human PCa cells. In particular, we verified if the invasive ability of an *in vivo* selected bone-seeking PCa cell line demonstrated molecular dependence on EGF-R activation.

Materials and Methods

Cell culture

The PC3 human prostate cancer cell line was originally obtained by ATCC (Rockville, MD, USA) and was maintained in DMEM supplemented with 10% fetal bovine serum, glutamine and penicillin-streptomycin (Sigma). PCb1 and PCb2 cell lines were obtained in our laboratory through serial selection of excised tumors with bone tropism in nude mice and were partially characterized in a previous study (Angelucci *et al.* 2004). Human stromal cells were isolated from surgical human bone specimens obtained by routine clinical practice with the informed consent of the patients. The medullar side of bone fragments was delicately scraped with a surgical blade and the recovered cellular material was resuspended in α -MEM containing antibiotics. Cells were repeatedly washed and filtered to eliminate cell clumps. Stromal cells were cultured in α -MEM supplemented with 20% fetal bovine serum, glutamine and penicillin-streptomycin (Sigma). The cells were quantified, and primary cultures were established at a plating density of 5×10^5 cells/cm² in α -MEM supplemented with 20% fetal bovine serum and antibiotics. The medium was changed initially at day 4 and then every other day thereafter until the cultures reached confluence. In order to evaluate cell proliferation, cells were plated at a density of 10^4 cells/cm² at various days of culture and were recovered with a solution of trypsin/EDTA, washed twice with ice cold PBS and counted in a Burkert slide. uPA was purchased from Chemicon International Inc. (Temecula, CA, USA). Mouse osteoblasts were isolated from calvaria of 7-day old CD1 mice. Bone were

cleaned free from soft tissues, and digested three times with 1 mg/ml *Chlostridium histolyticum* type IV collagenase and 0.25% trypsin, for 20 min at 37°C, with gentle agitation. Cells from the second and third digestion were plated and grown to confluence in DMEM plus 10% FBS. At confluence, cells were trypsinized by standard procedure and plated according to the experimental protocol. These cells expressed the osteoblast markers ALP, Runx-2, PTH/PTHrP receptor, type I collagen and osteocalcin.

Fluorescence analysis by FACS

Quantification of membrane-associated proteins was performed by flow cytometric analysis (FACScan; Becton–Dickinson, Mountain View, CA, USA). Cells were trypsinized, centrifuged, and left at 37°C for 1 h in DMEM/10% FCS in polypropylene tubes in order to reconstitute cellular external membrane. Cells were washed in saline buffer, fixed for 10 min at 4°C with a 4% buffered formalin solution, washed, and resuspended at 1×10^6 cells/ml. Pellets were incubated with 1 µg/ml primary antibody at 4°C for 1 h. Then, cells were washed twice and incubated with FITC-conjugated anti-mouse secondary antibody at 4°C for 1 h. Finally, after two more washes, cells were resuspended in 1 ml of phosphate buffer (PBS) and analyzed by flow cytometer. All the flow cytometric measurements were done under the same instrument settings, and at least 5000 cells were measured for each sample. Expression levels were evaluated as fluorescence index in the presence of the relevant antibody and reported as the ratio between this value and background staining of cells incubated with fluorescent secondary antibody only.

Animals and experimental *in vivo* models

Male CD1 nude mice (Charles River, Milan, Italy) were maintained under the guidelines established by our Institution (University of L'Aquila, Medical School and Science and Technology School Board Regulations, complying with the Italian government regulation n.116, January 27 1992 for the use of laboratory animals). Before any invasive manipulation, mice were anesthetized with a mixture of ketamine (25 mg/ml)/xylazine (5 mg/ml). Xenografts were obtained by injecting s.c. 1×10^6 tumor cells in 100 µl of 12 mg/ml Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). Tumor growth was monitored daily by measuring the average tumor diameter (two perpendicular axes of the tumor were measured by a caliper). The volume of the tumor was expressed in mm^3 according to the formula $4/3\pi r^3$. After 20 days, mice

were sacrificed by carbon dioxide inhalation and tumor mass was excised and weighed.

Heart injection of PCa cells was performed as previously described (Angelucci *et al.* 2004). Briefly, a 27 gauge needle on a tuberculin syringe containing 1×10^5 tumor cells in 0.1 ml of PBS was inserted in the second left intercostal space of four-week old nude mice. Animals were sacrificed by carbon dioxide inhalation 40 days after heart injections, or earlier if there were early signs of serious distress. The development of metastases was monitored weekly by radiography using a Faxitron cabinet x-ray system (Faxitron x-ray corp., Wheeling, IL, USA). Burden of osteolytic lesions was evaluated by digital examination of radiography (ImageJ, a public domain software by Wayne Rasband, NIH, USA). All animals were subjected to an accurate necroscopy and portions of various organs were processed for routine histological examination.

Real-time PCR

Total RNA was extracted from cultured cells using Genelute Mammalian Total RNA kit (Sigma) according to the manufacturer's protocol. To eliminate possible contamination by DNA, RNA samples were treated with 1 unit DNase I (Sigma). RNA was quantified by spectrophotometric analysis and 1 µg of RNA was used to synthesize cDNA (SuperScript III Platinum Kit, Invitrogen). Real-time PCR analysis was performed using Stratagene MX3000P personal Q-PCR (M-medical) in the presence of SYBR Green. The PCR reagents were provided in SuperScript III Platinum Kit (Invitrogen), and the conditions were chosen according to manufacturer's protocol. For all genes, 5 µl of cDNA were used except for GAPDH amplification which was performed using 2.5 µl of cDNA. Primers were designed using the online tool Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the sequences were as follows: EGF-R forward primer: 5'-TAATCTGTGTGTGCCCTGTA-3', reverse primer 5'-TTCCTTGATAAATTGGATGG-3'; HER2 forward primer: 5'-GGAGTCTTTGTGGATTCTGA-3', reverse primer: 5'-GGTCGCTTTTGTTCCTTAGAC-3'; uPA-R forward primer 5'-GCCTTACCGAGGTTGTGTGT-3', reverse primer 5'-CATCCAGGCACTGTTCTTCA-3' uPA forward primer: 5'-TGGTCTTTCTGGAGAGGTTA-3', reverse primer: 5'-CAGTGAGGATTGGATGAACT 3'; GAPDH forward primer: 5'-GGCCTCCAAGGAGTAAGACC-3', reverse primer: 5'-AGGGGTCTACATGGCAACTG-3'; RANKL forward primer: 5'-CCTTTTGCTCATCTCACTATT-3',

reverse primer: 5'-AATGTTGGCATAACAGGTA-ATA-3'; OPG forward primer: 5'-TGCTGTTC-CTACAAAGTTTACG-3', reverse primer: 5'-CTTTGAGTGCTTTAGTGCGTG-3'. Mean threshold cycle (Ct) values were determined by Strata-gene software using three distinct amplification curves for each gene. Relative expression of the target gene was estimated using the formula: relative expression = $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ (target gene) - C_t (GAPDH). The relative gene expressions are not comparable among different genes because the C_t (target gene) was multiplied by different factors.

Alu-PCR analysis

Alu elements are short repetitive sequences present only in the primate genome, reaching a copy number of over a million per genome. In order to detect human Alu, mice were sacrificed by carbon dioxide inhalation and the rear limbs were immediately processed. Bones were accurately deprived of surrounding soft tissues and opened with a surgical blade in order to expose the medulla. Bone fragments were incubated with 100 mM Tris-HCl pH 8.5, 0.5 M EDTA, 10% SDS, 5 M NaCl, 20 mg/ml proteinase K at 37 °C for 12 h. DNA was isolated by phenol/chloroform extraction, precipitated with ethanol and suspended in 0.1 M Tris-HCl pH 8.0, 0.5 M EDTA. After spectrophotometric quantification, DNA was subjected to 30 cycles of PCR amplification for Alu elements using the primers 5'-CGAGGCGG-GTGGATCATGAGGT-3' and 5'-TCTGTCGCCC-AGGCCGGA-3' (Roy-Engel *et al.* 2001) and the amplified sequence was visualized by 2% agarose gel electrophoresis.

Invasion assays

PVPF 8 µm polycarbonate filters (Nucleopore, Con-corezzo, Milan, Italy) were coated on one side with 250 µg/ml Matrigel, rinsed once with PBS and then placed in contact with the lower chamber containing chemoattractants. Cells (1×10^5 per chamber) were trypsinized, washed twice with PBS, rinsed in complete medium and incubated at 37 °C for 30 min to reconstitute the membrane structures and then added to the upper compartment of each chamber in medium without FCS. Cells were allowed to migrate through coated filters for 8 h. The cells attached on the lower membrane surfaces were stained with 0.1% crystal violet in 0.1 M borate, pH 9.0 and 2% ethanol for 20 min at room temperature. Cells were counted at $\times 400$ magnification in standard optical microscopy and the average number of cells per field in five

random fields was recorded. Triplicate filters were used and the experiments were repeated three times.

Western blotting and immunoprecipitation

Total cell lysates were obtained resuspending the cells in buffer containing 1% Triton, 0.1% SDS, 2 mM CaCl_2 , 100 µg/ml phenylmethyl sulfonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Hercules, CA, USA). 40 µg of proteins were electrophoresed in 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), which was then blocked overnight with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBS-T) containing 10% non-fat dry milk. The membrane was then incubated with 1 µg/ml of polyclonal anti-EGF, anti-p-EGF-R (Tyr 1173), uPA (H140) or MMP-9 (2C3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies in TBS-T and with specific horseradish peroxidase-conjugated secondary antibodies in TBS-T. Protein bands were visualized using a chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ, USA). For EGF-R immunoprecipitation, lysates with the same amount of protein in the same volume were incubated overnight at 48 °C with anti-EGF-R antibody, and protein-G-Sepharose beads (Calbiochem, San Diego, CA, USA) were then added for a further 2 h. After washing once with the cold lysis buffer and four-times with ice-cold PBS, the immunoprecipitates were then processed for detecting co-immunoprecipitated EGFR as described in this section.

Protease expression by zymography

Expression and activation of gelatinase B (pro-MMP-9) was analyzed by zymography performed using an SDS-polyacrylamide gel copolymerized with 0.1 mg/ml gelatine. For plasminogen activator analysis, gels were made by copolymerizing SDS-polyacrylamide with 0.1 mg/ml lactose-free casein and 15 mg/ml human plasminogen. Gels were washed three times with 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100 for 15 min under shaking conditions to remove SDS. Then gels were incubated in Tris-HCl (pH 7.4) containing 10 mM CaCl_2 , 200 mM NaCl (for gelatinases), or without salts (for plasminogen activators) at 37 °C. At the end of incubation, gels were fixed and stained with 0.1% Coomassie Blue solution. Enzyme-digested regions were identified as white bands against a blue background.

Figure 1 Metastatic ability of PC3 cell sub-lines. (a) Number of mice with metastases 40 days after intracardiac injection of the same amount (1×10^5) of PCa cells. (b) Representative radiographies of nude mice injected with 1×10^5 PCb2 cells showing preferential sites of bone metastases. Arrows indicate the osteolytic lesions derived from metastatic tumor activity. (c) The invasive ability of PC3 sub-lines are compared with parental cells in the presence of serum free medium (SFM), of 50 ng/ml EGF, and of bone stromal cell conditioned medium (BCM). The results are the mean of five independent experiments. The test was performed allowing cells to migrate across Matrigel for 8 h.

Statistical analysis

Results are expressed as means $\pm$ s.d. for at least three distinct experiments. Demonstration of significant differences among means were performed by Student's *t* analysis considering 0.05 the threshold value of *P*. Differences in the success rate between treatments *in vivo* were compared with χ^2 test for 2×2 tables. All statistical analyses were performed using Kaleidagraph 3.6 (Synergy Software, Reading, PA, USA).

Results

Selection of highly metastatic PC3 cell sub-lines

The injection of PC3 cells in the left ventricle of nude mice recapitulates the natural spreading of tumor cells through arterial circulation, leading to metastasis formation. In our model, the injection of 1×10^5 PC3 cells produced bone metastases in 70% of mice (Fig. 1a). Non-bone metastases were less frequent and involved primarily lungs and lymph-nodes. Bone metastases were induced starting from the third week after cell injection and reached a variable burden, causing after 5 weeks, the complete destruction of cortical bone in about 20% of mice. One tumor mass excised from bone metastasis with extracortical growth

was used to establish a new cell line termed PCb1. PCb2 cell line was obtained from a bone metastasis generated by PCb1 cells as previously described (Angelucci *et al.* 2004). In order to test their metastatic ability, the two PC3 cell sub-lines were injected in the heart with the same procedure used for PC3 cells. Results indicated that only PCb2 cells were able to form bone metastases in a greater percentage of mice compared to PC3 cells (Fig. 1a). Although PCb1 cells generated the same number of bone metastases as PC3 cells, we noticed that the bone osteolytic lesions were more uniformly distributed in the entire skeleton in comparison to parental cells that colonized preferentially at the rear leg. Bone metastases were visible as lytic lesions by X-ray scan and were localized primarily in the epiphysis of tibias and femurs (Fig. 1b). Other less common sites of lytic lesions were omerus, spine, and pelvis (Fig. 1b). PCb1 and PCb2 cells showed the same growth rate both in standard culture conditions and *in vivo* when injected s.c. (data not shown). The invasive potential of sub-lines was tested *in vitro* within the fifth cell culture passage. When cells were seeded for 8 h on Matrigel-coated filters, in absence of chemotactic gradient or serum, less than 10 cells were able to cross the Matrigel barrier without any significant difference among the three cells lines. Both in the presence of EGF and of bone stromal cell

Figure 2 Effect of gefitinib on PCa cells. (a) Relative levels of total and auto-phosphorylated EGF-R, MMP-9 and uPA in presence of three different concentrations of gefitinib (0.1, 0.5, 1 µM), serum-free medium (SFM), bone stromal cell conditioned medium (BCM). (b) Growth curves of PCa cells cultured in presence of 50 ng/ml EGF (control) or of 50 ng/ml EGF plus 60 µg/ml gefitinib. The concentration of gefitinib was chosen calculating the mean of the three different IC50 (table, inset). The results are the mean of three independent experiments. * P (PC3 vs PCb1) and P (PC3 vs PCb2) > 0.1 (c) 0.5 µg/ml gefitinib (black columns) was added to PC3 and PCb2 cells 30 min before the invasion test and during the experiment. Invasion assay was performed allowing cells to migrate across a Matrigel coated filter. Cells were counted after 8 h and the chemoattractant used was BCM. The results are the mean of five independent experiments. * P < 0.01. (d) MMP-9 and uPA activity by zymography in PC3 and PCb2 conditioned media recovered after 48 h of incubation in the presence of BCM and two concentrations of gefitinib (0.5 and 1 µM).

conditioned medium (BCM), we observed an increase of about 4-fold in invaded PC3 cells in comparison to basal conditions. PCb1 and PCb2 cells showed a progressive increase in their invasive ability in the presence of EGF and BCM. The difference when compared to PC3 cells was particularly evident in the presence of BCM. As shown in Fig. 1c about 50% more of PCb2 cells were invaded, compared to PC3 cells and were counted in the opposite surface of Matrigel-coated filters.

The effect of gefitinib on *in vitro* PCa cell proliferation and invasion

The addition of different doses of gefitinib in the range of 0.1–1 µM inhibited, in a dose dependent manner, the EGF-R autophosphorylation in PC3 cells compared

with total EGF-R (Fig. 2a). Cells were treated with gefitinib in the presence of 50 ng/ml EGF in serum-free medium (SFM) and the blockade of EGF-R activation was observed within 30 min. An increment in the presence of the phosphorylated form of EGF-R, but not in its total form, was observed not only in the presence of EGF but also when cells were cultured in SFM plus 30% of BCM (Fig. 2a). The effect was observed 24 h after the cell starvation and within 4 h after the addition of BCM. When we analyzed the presence of proteolytic enzymes in conditioned medium of PC3 after 24 h of treatment with EGF or BCM, we observed an increment in the secreted metalloprotease-9 (MMP-9) and uPA. The presence of increasing doses of gefitinib was able to markedly reduce the presence of MMP-9 and uPA in conditioned medium (Fig. 2a).

Figure 3 Inhibition of xenograft growth by gefitinib. (a) Mice were inoculated s.c. with PC3 or PCb2 cells and were divided in two groups: one received oral administration of drug vehicle, and the other received 150 mg/kg of gefitinib. The drug and the vehicle alone were given in the days indicated by white arrows. Tumor volume was measured at days 7, 14 and 21. The results are the mean of three independent experiments. $*P > 0.05$. (b) At the 21st day animals were sacrificed and tumors were excised and weighed. Histograms show the mean reduction in tumor mass weight. Photos of four representative tumors for each cell line in control and in treated groups are shown.

PCa cells showed a significant reduction in their proliferative capacity 72 h after the treatment with 0.5 μ M gefitinib, reaching 50% inhibition after 96 h (Fig. 2b). The gefitinib did not generate appreciable differences in the proliferative response among different PCa cell lines as demonstrated also by the IC_{50} analysis (Fig. 2b, table inset).

In order to test the effect of gefitinib on the invasion of PCa cells, we pre-treated PC3 and PCb2 cells with gefitinib for 1 h before the invasion assay and added gefitinib in the upper compartment of the Boyden chambers. In Figure 2c we show the inhibition of BCM-mediated invasion by 0.5 μ M gefitinib. In order to evaluate if the pro-invasive effect of BCM was dependent on the functionality of EGF-R, we pre-treated PCa cells with 0.5 μ M gefitinib and then seeded cells in the presence of BSM in a Boyden chamber. Both PC3 and PCb2 cells matched with gefitinib showed a reduced ability to degrade and invade Matrigel in the presence of BCM, but while the inhibition in PC3 cells was about 30% compared to control. In PCb2 cells, we observed a more evident effect generating a reduction of about 50% in invaded cells relative to control. When we evaluated the proteolytic activity in conditioned media from PC3

and PCb2 cells treated with gefitinib for 24 h we observed a decrease in both gelatinolytic and caseinolytic activity, with the higher effect observed for uPA secretion by PCb2 cells.

Gefitinib inhibition of the *in vivo* PCa cell proliferation

In order to generate tumor xenografts, PC3 and PCb2 cells were injected s.c. with Matrigel in nude mice. Tumor volume was monitored weekly and the results were expressed as mean values. When we injected an equal number of PC3 and PCb2 cells, the growth rates of cell lines were very similar (Fig. 3a). Mice did not show any sign of distress until the end point, and necropsy did not reveal the presence of metastases. Ten mice per cell line were randomly selected among twenty and each underwent treatment with 150 mg/kg gefitinib for 4 cycles of 5 consecutive days, separated by 2 days without drug administration. Control groups received the vehicle according the same schedule (Fig. 3a). After three cycles of treatment, at day 21 we sacrificed the animals and measured tumor volume and weight. Groups treated with gefitinib revealed a significant reduction in tumor volume appreciable

Table 1 Inhibitory effect of gefitinib on metastasis formation

Treatment group	Body weight (g)	Metastases (n/total number of mice)		Metastases (% reduction)	
		Bone (%)	Other sites (%)	Bone	Other sites
PC3	20	12/16 (75)	4/16 (25)	–	–
PC3+gefitinib	17	8/20 (40)	2/20 (10)	47% (a)	60%
PCb2	20	16/18 (89)	3/18 (17)	–	–
PCb2+gefitinib	18	3/18 (17)	1/18 (6)	81% (b)	65%

(a) $P = 0.037$; (b) $P < 0.001$. Mice were inoculated by heart injection with PC3 or PCb2 cells and were divided in two groups: one received oral administration of drug vehicle, and the other received 150 mg/kg of gefitinib. The drug and the vehicle alone were given in 4 cycles of 5 days with two days without drug, starting from the second day after tumor cell injection. Privileged sites of metastasis other than bone were lung and abdominal lymph nodes. Number of metastases and their reduction in presence of gefitinib are indicated.

from day 10 and reaching an end point value of 50% compared to the untreated groups. However, the response of PC3 and PCb2 cells to gefitinib did not differ and the mean volume of tumors treated with the drugs were very similar along the entire experiment. The same result was obtained measuring tumor weight (Fig. 3b). In the bottom panel of figure 3b, we show representative images of two tumors excised from each experimental group.

Reduction of experimental bone metastasis by gefitinib

Next we evaluated the effectiveness of gefitinib in metastatic blockade by using the heart injection experimental model. Mice were divided in four groups according to the type of cell line inoculated (PC3 or PCb2) and of the treatment (drug vehicle or 150 mg/kg gefitinib). Mice receiving gefitinib did not show any additional signs of distress compared to the control group, except for a statistically not significant reduction in the median body weight. At the end point, 5 weeks after cardiac injection, the overall number of metastases by PC3 cells in the gefitinib treated group was about half of the control group. In fact, bone metastases were visible by radiography only in 8/20 mice, while necroscopic analysis revealed the presence of metastatic foci in non-bone sites only in 2/20 mice vs 4/16 of the control group (Table 1). As expected, PCb2 cells were able to generate a higher number of bone metastases compared to PC3 cells. Mice with at least one evidence of bone lesion were about the 90% of total inoculated mice. The treatment with gefitinib demonstrated to be very effective in the reduction of bone metastases generated by PCb2 cells, which developed in less than 20% of the mice (Table 1). Although PC3 and its sub-lines caused severe osteolytic metastases *in vivo*, their conditioned medium or

co-culture had no effect on maturation of mouse osteoclasts from precursor cells *in vitro* (data not shown) (Inoue *et al.* 2005). In order to address the role of osteoblasts in the gefitinib induced reduction of lytic lesions, we performed primary culture from mouse bone marrow and evaluated the mRNA expression levels of the receptor activator of Nf-kappa-B ligand (RANKL, osteoclastogenesis inducer) and osteoprotegerin (OPG, osteoclastogenesis repressor) in osteoblasts (Fig. 4a). Mouse osteoblasts were treated with gefitinib or with conditioned medium of PC3 and PCb2 cells cultured with or without gefitinib. Conditioned media from PCa cells determined a strong upregulation in RANKL mRNA, particularly in PCb2 cells. Gefitinib determined a significant reduction in the capacity of PCa conditioned medium to induce the expression of RANKL, while the direct treatment of osteoblasts did not induce any significant change in RANKL levels. Expression levels in OPG mRNA were always lower in basal conditions but the different treatments failed to show significant differences. Since all mice bearing bone metastases showed lytic lesions in rear limbs, we planned to verify the presence of PCa cells in femur and tibia also by a more sensitive method. Bone medulla of control and treated animals were recovered and analysed for the presence of human Alu sequence by PCR amplification and gel electrophoresis. The same procedure, performed using serial dilution of PC3 cells injected directly in mouse bone medulla, demonstrated that the technique allowed us to reveal as little as 1000 human cells (data not shown). Alu analysis showed the presence in bone medulla of PC3 cells in about 85% and of PCb2 cells in more than 90% of control mice, with an increment of 15% and 5%, respectively in positive mice compared with the X-ray analysis (Fig. 4b). By carrying out digital analysis of X-ray films, we measured the extension of the lytic lesions present in the corresponding legs. It was

Figure 4 Comparative analysis of bone metastasis formation between control and in gefitinib treated mice. (a) RANKL and OPG relative mRNA expression levels in mouse osteoblasts by quantitative-PCR. Osteoblasts were incubated with conditioned medium (CM) from PC3 and PCb2 cells cultured for 48 h with or without 0.5 μ M gefitinib. $P(A \text{ vs } B) < 0.05$. (b) Number of Alu-positive mice and mean extension ($\pm$ s.d.) of lytic lesions in rear limbs for each group of treatment. Radiographs of tibia and femur were analyzed by software elaboration in order to measure the extension of lytic lesions expressed in mm^2 . (c) Representative results from 9 different limbs of PCb2 cell injected mice. In the presence of lytic lesions both in tibia and femur the total burden was summed. The same bones were subjected to DNA extraction from bone medulla. DNA from adjacent tibia and femur were pooled and subjected to PCR analysis for the presence of the Alu sequences.

observed that gefitinib was also effective in the reduction of lytic burden. This phenomenon was particularly evident in PCb2 injected mice because they usually developed larger lytic lesions than PC3 injected ones (Fig. 4b). In Fig. 4c we show a comparative analysis between representative results obtained by X-ray and Alu-PCR analysis of PCb2 generated metastases. While all X-ray positive mice resulted also positive for the presence of PCb2 cells, two X-ray negative mice showed detectable levels of Alu-sequences (lane 1 and 3, Fig. 4c).

Molecular characterization

In order to explain the differential response of PCb2 cells to gefitinib relative to parental cells, we investigated the expression level of key molecular determinants involved in EGF-R activation. First, we performed a relative quantification of mRNA expression by real-time PCR using primers specific for EGF-R, HER2, uPAR and uPA (Table 2). EGF-R mRNA was detected at the same level in all PCa cell lines while HER2 mRNA was significantly less expressed in PCb2 cells compared to parental cells. On the contrary, we observed a significant increment in both uPAR and uPA expression in PCb2 cells compared to PC3 cells. PCb1 cells showed an intermediate phenotype expressing more uPA-R, but not uPA mRNA compared to PC3 cells. In order to confirm these results, we analyzed this expression also at the protein level. In particular we examined the presence on the plasma membrane of EGF-R, HER2, uPA-R and uPA by cytofluorimetric analysis (Fig. 5). Data obtained by real-time PCR were substantially confirmed with a significant increase in uPA-R and uPA expression as shown in the representative experiment (Fig. 5a, lower panel). As indicated by statistical analysis, PCb2 cells expressed significantly less EGF-R and HER2 than PC3 cells. In order to evaluate a possible interaction between uPA-R and EGF-R, we first verified the capacity of exogenous uPA to activate EGF-R. PC3 cells were starved for 24 h and cell lysates were extracted after a short incubation (30 min) with uPA in serum-free medium (SFM). The addition of 50 μ g/ml uPA determined a strong activation of EGF-R when compared with SFM. Then we immunoprecipitated EGF-R and examined the expression levels of p-EGFR and uPA-R. PCa cells were treated with BCM for 4 h prior to protein extraction. In PCb2 cells, a higher amount of uPA interacted with EGF-R compared to PC3 cells. Moreover, the complex observed in PCb2 contained mainly EGF-R in the active form.

Discussion

The progression of PCa implies that the acquisition of several new invasion abilities by cancer cells according to modalities and timing, are not easily predicted by clinical investigation. For this reason, it is very difficult to individualize an effective therapeutic approach, enabling the complete eradication of a tumor that has progressed to an advanced stage. Moreover, experimental models based on commercial cell lines fail in many cases to resemble the fundamental clinical characteristics of tumors. One of the most challenging

Table 2 Relative gene expression at the level of mRNA by quantitative-PCR analysis in PC3 cell line and its sub-lines

Gene	Relative expression (s.d.)			P-values	
	PC3	PCb1	PCb2	(a)	(b)
EGF-R	3.52 (0.24)	3.23 (0.32)	2.80 (0.25)	0.57	0.08
HER2	1.81 (0.13)	1.84 (0.21)	1.43 (0.18)	0.83	0.03
uPA-R	2.51 (0.65)	4.52 (0.71)	11.92 (0.83)	<0.01	<0.01
uPA	0.63 (0.04)	0.82 (0.05)	2.03 (0.02)	0.10	<0.01

(a) PC3 vs PCb1; (b) PC3 vs PCb2.

a

Protein	Fluorescence Index (S.D.)			P (#)
	PC3	PCb1	PCb2	
EGF-R	2.4 (0.9)	2.2 (0.6)	1.8 (0.6)	0.01
HER2	1.5 (0.5)	1.7 (0.8)	1.0 (0.5)	0.05
uPA-R	1.7 (0.7)	3.5 (0.8)	7.0 (1.5)	<0.01
uPA	2.1 (0.5)	2.5 (0.5)	5.7 (0.9)	<0.01

= PC3 vs PCb2

b

c

Figure 5 EGFR and uPAR in PC3 cell line and its sub-lines. (a) Expression on the cellular surface by cytofluorimetric analysis. The fluorescence index, calculated as described in Methods and materials, represents the mean of three different analyses. Fluorescence patterns obtained in PC3 and PCb2 cells using anti-uPA-R antibody are shown in the lower panel. White peak represents the auto-fluorescence (auto) observed when cells were treated only with the secondary antibody. (b) Relative levels of total and auto-phosphorylated EGF-R in presence of serum-free medium (SFM) and SFM plus 50 ng/ml uPA. (c) PC3 and PCb2 cells were treated for 4 h with BCM. EGFR was immunoprecipitated (IP) and the immune complex analyzed by immunoblot (IB) for EGFR total mass, phosphorylated EGFR and uPAR.

difficulties in tumor progression modelling is represented by the generation of an adequate pattern of metastases. The reasons are several, including an inadequate molecular asset acquired by tumor cells through their *in vitro* culturing. Once injected in mice these cells frequently fail to form visible tumors in short time, often entering into a phase of dormancy (Aguirre-Ghiso *et al.* 2003). Our experience has demonstrated that the highly aggressive potential of the PC3 cell line was easily overcome by sub-lines selected by their ability to colonize bone. The propensity of PC3 cells to form lytic lesions especially in rear limbs demonstrated that anatomical features also represent an important co-factor in the bone colonizing ability of tumor cells (Yoneda 1997). However this characteristic was partially lost by the first derived sub-line PCb1, that was able to metastasize in several different bone sites, demonstrating a more 'pure' molecular bone tropism. We hypothesized that PC3 sub-lines, during the acquisition of increased metastatic potential might be selected for the expression of specific molecular determinants that are already present in the parental cell line.

The PC3 cell line was originally derived from a bone metastasis and since it lacks the expression of the androgen receptor, it represents the prototype of hormone-insensitive PCas of poor prognosis. The discovery of new molecular targets controlling different proliferative cellular pathways from AR signaling, may offer new hopes for the rational design of an effective therapy for this kind of PCa. The activation of EGF-R triggers a complex network of downstream cellular pathways, modulating different cellular responses including apoptosis, angiogenesis, adhesion and motility. In effect, EGF-R inhibitors, including gefitinib, were able, as described by our research group and by other authors, to reduce the growth of PCa cells both *in vivo* and *in vitro*. As demonstrated by xenograft experiments, the highly selective metastatic sub-line PCb2 did not show any significant difference in its *in vivo* proliferative capacity in response to gefitinib compared to the original PC3 cell line. On the contrary, we observed that PCb2 cells were particularly responsive to gefitinib in their invasive and metastatic ability. The acquisition of a new dependence on EGF-R in PCb2 cells was gained in absence of an upregulation of EGF-R or HER2 expression. The analysis of HER2 was performed because the over-expression of EGF-R-homologous HER2 receptor, which is highly expressed in several human cancers, can potentiate EGF-R function by: increasing EGF binding affinity, stabilizing and recycling EGF-R-HER2 heterodimers; expanding the repertoire of

receptor-associated substrates and signaling responses (Karunagaran *et al.* 1995, Worthylake *et al.* 1999). Although our data show that HER2 expression is not appreciably modulated in highly metastatic PCa cells, suggesting that HER2 over-expression is not a driven event during the acquisition of bone tropism. We can not exclude that targeting HER2 may be useful in determining a further reduction in the number of metastases. We hypothesized that the gain of function observed in PCb2 cells was mainly due to the expression of key molecular determinants other than EGF-R and was able to direct its activation to different functions. Increasing evidence suggests that many of the cellular responses triggered by EGF-R are not only activated by EGF or TGF- α , but also by association with intracellular signal transduction factors or membrane associated proteins. The interaction with specific components probably determines the nature of the downstream cellular effects generated by EGF-R activation. One emerging candidate in modulating the response through EGF-R activation is uPA-R. The assembly of a multimeric complex formed by uPA, uPA-R, integrins and EGF-R is a predictor of *in vivo* behaviour of different carcinomas including PCa (Aguirre-Ghiso *et al.* 2003). In our work we demonstrated that exogenous uPA was able to induce EGF-R autophosphorylation in the absence of known EGF-R ligands. The transactivation of EGF-R by uPA-R was already shown in breast cancer, where interestingly it appears to mediate cellular invasiveness rather than proliferation (Guerrero *et al.* 2004). In addition, as we previously demonstrated using the same experimental model, the inhibition of uPA-R expression, through the administration of antisense oligo anti-uPAR, significantly influences the production of bone metastases by PC3 cells (Margheri *et al.* 2005). The activation of EGF-R by uPA may be facilitated by the formation of complexes containing uPA-R. These complexes in the highly metastatic cell line PCb2 are more abundant and facilitate the activation of EGF-R. Since gefitinib was able to preferentially block metastatic dissemination, the association between EGF-R and uPA-R seems to represent an important determinant in a specific phase of disease progression. In particular, the functional interactions between these two receptors raise the possibility that extensive influence in bone homing and remodelling may occur in PCa cells. Several factors playing a key role in normal bone remodelling have been described to also determine the predilection of PCa cells for the bone microenvironment, namely EGF, TGF- α and uPA (Daci *et al.* 1999, Cooper *et al.* 2003). The finding that bone is a potential storehouse

of growth factors and that PCa cells in culture respond to bone-derived growth factors, suggests that the release of these factors during metastatic bone remodelling may induce a positive feedback mechanism in tumor growth. PC3 cell line and its sub-lines failed to induce *in vitro* osteoclastogenesis. On the contrary, the activation of osteoclasts and the subsequent induction of bone resorption induced by tumor cells are thought to depend on the balance of the local RANKL-OPG ratio determined by osteoblasts activity. Both PC3 and the highly metastatic PCb2 cell lines are able, through the release of soluble factors, to induce a strong overexpression in RANKL mRNA in mouse osteoblasts. So our data suggest that the action of gefitinib in the bone microenvironment depends on a dual mechanism: direct inhibition of tumor cell proliferation and blockade in the cross-talk between tumor and bone cells. Recent work by Normanno *et al.* (2005) demonstrated that gefitinib may play a role in osteoclastogenesis inhibiting EGF-R present in continuous stromal-like cell lines. This mechanism could also be important in our experimental model, although our data indicates that gefitinib acts mainly through the modulation of factors released by tumor cells. Among these factors, which need further investigation, may also include EGF-R ligands secreted by PCa cells. However, we did not observe any direct effect of gefitinib on mouse osteoblasts *in vitro*.

The analysis of Alu sequences in metastatic sites allowed us to determine the presence of silent micro-metastases that do not influence bone remodelling, since it is not visible by radiography. Our results showed the concomitant absence of tumor cells and of osteolytic lesions in gefitinib-treated mice, again demonstrating the dual role of EGF-R inhibition. We thus hypothesized that gefitinib blocked bone tropism of PCa cells in a very early phase of metastatic colonization, preventing the gain of a critical tumor mass in the target organ. The presence of a limiting number of tumor cells was due to a blockade in the establishment of the necessary crosstalk between tumor and bone cells. This could be supported by the establishment of a positive feedback mechanism based on reinforcing an interaction between EGF-R and uPA-R. It has been demonstrated that the expression of uPA and uPAR in PCa cells is a necessary condition for determining the invasive ability downstream from EGF-R signaling (Mamoune *et al.* 2004, Festuccia *et al.* 2005a), indicating the existence of a possible additive mechanisms regulating PCa progression. In particular, the modulation of uPA secretion and activation may play a key role for determining the ability

of tumor cells to 'seed' in the bone tissue. Moreover the importance of proteases in the initial stages of tumor implantation in the bone is linked, in addition to the migratory capacity, to the tumor potential in remodelling the bone matrix (Nemeth *et al.* 2002).

In conclusion, our study demonstrates that the EGF-R tyrosin-kinase inhibitor, gefitinib, is an effective drug in the treatment of the metastatic stages of PCa. Although the specific mechanism of action is not completely understood, gefitinib appears to target the different aspects of the complex molecular processes responsible for bone homing. In particular, our results suggest that even metastatic tumor cell lines retain *in vivo* a degree of dependence on surface receptors like EGF-R and uPA-R. This observation provides new therapeutic opportunities in controlling the spread of a tumor. This characteristic indicates that gefitinib may be suitable for the clinical control of the early micro-dissemination of PCa tumor cells.

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