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MAMMARY CARCINOMA ASSOCIATED FIBROBLASTS AS KEY PLAYERS IN THE ACQUISITION OF HORMONE INDEPENDENCE

INTERACTION BETWEEN PROGESTERONE RECEPTORS AND FIBROBLAST GROWTH FACTOR RECEPTOR 2

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Abstract We have developed an experimental model of mouse mammary carcinomas expressing high levels of hormone receptors which transit through different stages of hormone dependence. In this study we have demonstrated that carcinoma associated fibroblasts (CAF) from hormone independent tumors (HI) express higher levels of FGF-2 than those of hormone-dependent tumors. Stromal FGF-2 activates FGFR-2 in the epithelial cells which in turn activates progesterone receptors (PR) and induces cell proliferation. In addition we showed nuclear interaction of FGFR-2 and PR suggesting novel cross talk mechanisms between both pathways. These results were reproduced in the T47D human breast cancer cells. Our data indicate that stroma participates in HI tumor growth and that the FGF-2- FGFR-2 axis should be considered as a therapeutic target together with antiprogestins in PR positive breast cancer.

Key words: carcinoma associated fibroblasts, fibroblast growth factor 2, FGF receptors, hormone dependence, mammary carcinoma, progesterone receptors

Resumen ***Rol primordial de los fibroblastos asociados al cáncer de mama en la adquisición de la hormono-independencia.*** Hemos desarrollado un modelo experimental de cáncer de mama en ratones en el cual los tumores expresan alto contenido de receptores hormonales y transitan por distintos estadios de hormono-dependencia. Hemos estudiado el rol de los fibroblastos asociados a tumor (CAF) en el crecimiento hormono-independiente. En este trabajo mostramos que los CAF de tumores hormono-independientes (HI) expresan mayores niveles de FGF-2 que los CAF de tumores hormono-dependientes. El FGF-2 estromal activa a los RFGF-2 de las células epiteliales y a su vez esta interacción activaría a los receptores de progesterona (RP) estimulando así la proliferación celular. Demostramos también interacción nuclear directa de RFGF-2 y RP sugiriendo nuevas formas de conversación cruzada entre ambas vías. Los resultados en el modelo experimental fueron reproducibles en la línea celular de cáncer de mama humano T47D. Estos resultados sugieren que el estroma participa en el crecimiento HI y que la vía FGF-2- FGFR-2 constituye un blanco terapéutico que debe ser tenido en cuenta para ser usado en conjunción con antiprogestágenos en cáncer de mama RP positivo.

Palabras clave: fibroblastos asociados a tumor, factor de crecimiento fibroblástico 2, receptores de progesterona, dependencia hormonal, cancer de mama

Breast cancer is the most frequent cancer in women from industrialized countries and second only to lung cancer as a cause of death[1]. For this particular type of cancer, most changes in tumor behavior and progression have, for years, largely been associated with progressive series of intrinsic changes in the malignant epithelial cells, while a role for supporting structures and the mesenchymal component was overlooked. Starting in the early 70´s with Folkman's seminal paper[2] that highlighted the essential role of blood vessels in tumor development and growth, there has been increasing interest in the characterization of tumor stroma. Several stromal cells among them fibroblasts have been demonstrated to be able to synthesize and release a wide range of growth factors. A growth modulatory role for stromal components in breast cancer has been recently demonstrated by Orimo et al[3], who showed that cancer-associated fibroblasts (CAF) inoculated together with MCF-7 cells increased tumor growth. These reciprocal interactions between epithelial and stromal cells have been shown by many to be key determinants in morphogenesis, proliferation and differentiation of both endocrine and non-endocrine target organs. Moreover, it has also been shown that stromal al-

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terations may play a significant role in carcinogenesis and the changes induced may even precede the malignant conversion of epithelial cells[4, 5].

Progestins and breast cancer

Unlike estrogens, the role of progestins in breast cancer has, until recently, been underestimated. Following the uterus model, progesterone has been exclusively regarded as a differentiating agent opposing the proliferative effects of estrogens. This, coupled with the fact that estrogen metabolites have experimentally been shown to be carcinogenic, as well as the therapeutic success of antiestrogens in breast cancer and the stimulatory effects of estrogens on most human breast cancer cell lines, may explain that progestins have been relegated to a secondary role in the development of breast cancer. However, an increasing body of experimental evidence indicates that they play an important role in the induction and maintenance of the neoplastic phenotype in the mammary gland[6-8]. The relevance of such data has been underlined by the findings of the Women's Health Initiative trials[9], as well as by other epidemiological studies that have reported that estrogen plus progestin, but not estrogen alone, is associated with a greater risk of breast cancer.

The FGF-2-FGFR-2 axis

Fibroblast growth factor-2 (FGF-2) is the prototype member of a large family of structurally-related, heparin binding, polypeptide growth factors found in virtually all tissues studied, both normal and malignant, which transduce signals through cell-surface transmembrane tyrosine kinase receptors (RTK), that can regulate cell growth, migration, differentiation or survival. FGF-2 binds preferentially to its receptor (FGFR-2). The FGF-FGFR pathway has acquired increased attention in the last years, not only in development and in endocrine or bone disorders, but also in cancer research[10]. Controversial data have been reported regarding the role of FGF-2 in tumor growth. Several studies point to a decrease in FGF-2 expression during neoplastic transformation, while others, to increases in FGF-2 expression in patients bearing breast tumors. *In vitro*, also proliferative or inhibitory effects have been ascribed for FGF-2; depending on the cell type, the extra matrix cell components or the cell cycle stage, they may exert different functions. The preferential stromal localization of FGF-2 has already been described in prostatic carcinomas and in human breast cancer. Initially, FGFR-3 and c-erbB4 have been postulated as the transmembrane RTKs which experienced nuclear translocation, but later, it has been proposed that this could be a more general mechanism by which membrane receptors such as EGF-R could act as transcription factors[11]. Nuclear localization of FGFR may reflect ligand activation: vesicles containing activated FGFR are internalized and then translocated through the nuclear membrane[12].

The MPA breast cancer model

We have developed a model of hormonal carcinogenesis in which the administration of medroxyprogesterone acetate (MPA) to female virgin BALB/c mice leads to the development of ductal metastatic mammary carcinomas expressing estrogen (ER) and progesterone receptors (PR). These tumors are maintained through syngeneic passages in MPA-treated animals to preserve their original hormone-dependent (HD) growth behavior. Hormone-independent (HI) lesions may arise either spontaneously or by selection, and are able to grow in untreated ovariecto-mized animals. All HD and HI tumors express ERα and the classical PR_A and PR_B isoforms of 83 kDa and 115 kDa, respectively, as well as a novel band of 78 kDa[13]. This is one of the few breast cancer models in which the tumors share most of the features of human breast cancer: ductal histology, metastatic ability, expression of ER and PR and transit through different stages of hormone responsiveness. Thus, it is especially useful for studies of hormone receptor function, hormone responsiveness and tumor regression. Using *in vivo* approaches we have demonstrated that these tumors are inhibited by anti-progestins, estrogens and tamoxifen[14]. In *in vitro* primary cultures of C4-HD, we have demonstrated a direct proliferative effect of MPA, which is mediated by PR[15]. FGF-2 had the same proliferative effect as MPA. Antiprogestins (mifepristone/RU 486 and onapristone/ZK 98,299) and PR antisense oligodeoxynucleotides (asPR) inhibited progestin or FGF-2 induced cell proliferation (Fig. 1 A), thus showing that PR are involved in cell growth and may be activated by ligand independent mechanisms[16].

Working hypothesis

Tumor stromal fibroblasts, usually referred to as carcinoma-associated fibroblasts (CAF), may secrete factors such as FGF-2, which, acting in a paracrine fashion, may activate their cognate receptors (FGFR) in the epithelial cells and, in turn, activate PR to stimulate HI tumor growth.

Co-cultures

We have developed primary cultures of epithelial (EPI) cells or fibroblasts (CAF) from HD and HI murine mammary carcinomas with purity higher than 95%. An interesting fact was that EPI from HI tumors grew very well in the presence of 10% fetal calf serum, but not when

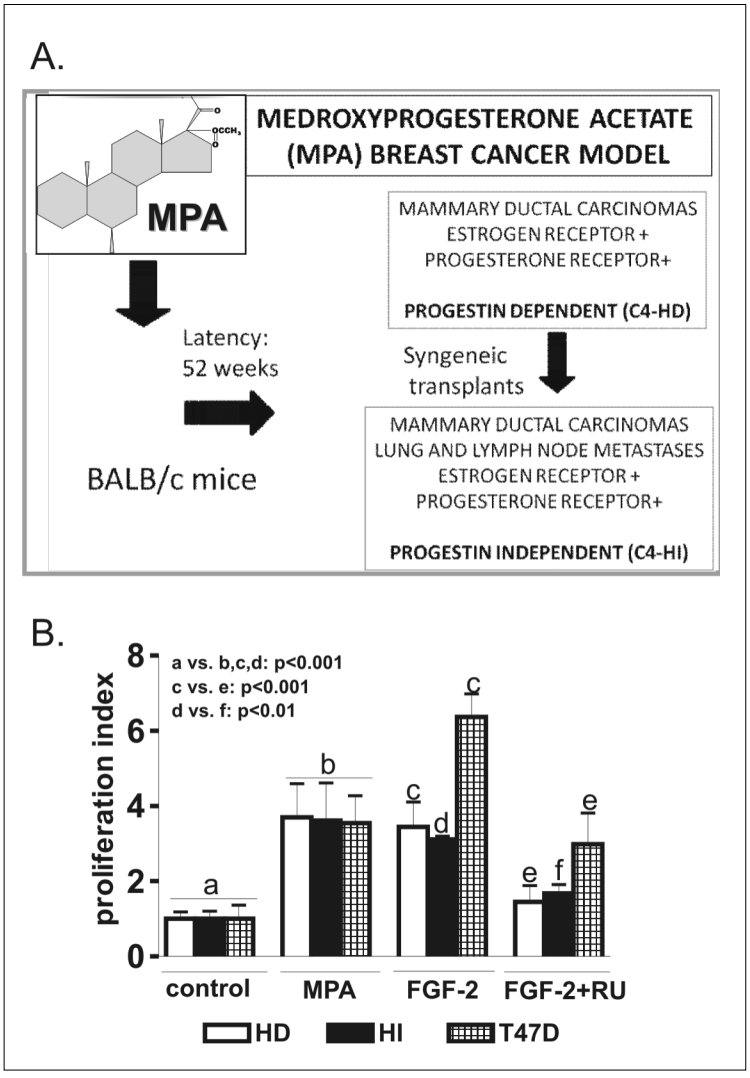

Fig. 1.– A. MPA breast cancer model. MPA induces ductal mammary carcinomas in BALB/c female mice which are maintained by syngeneic transplantation in MPA-treated mice. These MPA-dependent tumors express high levels of ER and PR. Hormone-independent variants may be generated by tumor transplantation in untreated animals. Most of these tumor variants still express high levels of ER and PR, are highly metastatic and still respond to an endocrine therapy. B. Effect of MPA, RU 486 and FGF-2 on C4 mouse mammary tumor cells and T47D human breast cancer cells in cell proliferation (^{3}H-thymidine uptake). Primary cultures of purified epithelial cells from C4-HD or C4-HI tumors or T47D human breast cancer cells were cultured in 96-well microplates. After attachment the medium was replaced by 1% chFCS. The cells were treated during 48 hr with MPA 10^{-8} M or FGF-2 50 ng/ml, with or without RU 486 (RU) 10^{-8} M and a pulse of ^{3}H-thymidine was added in the last 20 hr. Proliferation index was calculated as experimental cpm/control cpm, and a representative experiment of other 3, using octuplicates in each experiment, is shown.

cultured in the presence of 1% charcoalized fetal calf serum (chFCS) or without serum unless the cultures were co-cultured with CAF. A similar finding[17] was observed using the epithelial cell line MC4-L4, that doesn't grow in the absence of serum unless co-cultivated with a fibroblastic tumor stromal cell line, the MC-L4F. We decided to further explore this phenomenon using co-cultures of EPI from HD tumors (EPI-HD) or from HI tumors (EPI-HI) with either CAF-HD or CAF-HI and evaluating cell proliferation by ^{3}H-thymidine uptake. CAF-HI induced a higher epithelial (as confirmed by counting cytokeratin-positive cells) proliferative effect than CAF-HD when cultured with EPI-HD or EPI-HI. In addition, the growth of EPI-HI + CAF HI cultures was inhibited by RU 486, as well as by a neutralizing antibody against FGF-2 (recently developed by A. Baldi, IBYME). As evaluated by bromo-deoxyuridine (BrdU) and cytokeratin immunostaining, both treatments affected the epithelial compartment exclusively

(not shown). EPI-HI cells *in vitro*, gave the same response to MPA and FGF-2 as to C4-HD cells (Fig. 1 A). These results suggested that a) CAF-HD and CAF-HI are different; b) FGF-2 is a likely candidate for the induction of EPI-HI growth and c) PR from EPI-HI are key molecules driving cell proliferation.

Differences between CAF-HD and CAF-HI

In a previous report we have shown that FGF-1 and FGF-2 had stimulatory effects similar to those of MPA on EPI-HD cells[16], and that the proliferative effect was abolished by two different antiprogestins, mifepristone and onapristone, and by asPR. On this basis, we have postulated that a cross talk exists between the FGF and PR pathways[16], and that FGF-2 is a stromal growth stimulating candidate that could replace progestins in HI tumors. FGFs bind mainly to 4 FGF receptors[10, 18], some of which have been exclusively found in epithelial cells. FGF-2 is expressed mainly in the stromal compartment of a C4-HI tumor section, while FGFR-2 is positive in malignant epithelial cells. Moreover, we demonstrated a 20 fold increase in FGF-2 expression in CAF-HI ($p<0.001$, $n=5$) as compared with CAF-HD, as well as increased levels of FGFR-2 in HI tumors as compared with HD untreated tumors ($p<0.001$). MPA-treatment induced an increase in FGFR expression. These results suggest the existence of a paracrine loop between the stromal and epithelial compartments, in which FGF-2 may act as a growth stimulating factor in HI tumors. Interestingly, exogenous addition of FGF-2 to EPI-HD increased PR-DNA binding and PR phosphorylation as effectively as MPA, suggesting that FGF-2 may increase PR activation. Taken together, these results indicate that FGF-2 is a stromal factor in the HI stroma that participates in the increase of cell proliferation induced by CAF-HI and that this can occur because it induces PR activation.

CAF-HI induces PR phosphorylation and PR-DNA binding

As expected, incubation of EPI cells with CAF-HI induced the activation of PR, as evaluated by western blots using 2 different pPR antibodies, and in accordance, they increased PR-DNA binding. CAF-HI were better partners than CAF-HD. The use of a neutralizing FGF-2 antibody or siRNA to FGFR-2 transfected epithelial cells to block the FGF-2- FGFR-2 pathway inhibited CAF-HI induced cell proliferation.

FGF-2 increases FGFR-2 and PR nuclear interactions

This relationship between PR and FGFR pathways led us to study the interaction of both receptors. In cell cultures, FGFR-2 co-localizes with PR at the cell nuclei. Incubation of EPI with FGF-2 induced an increase in nuclear PR localization and co-localization of PR with FGFR-2. This effect is evident after 20 min of incubation and is greater after one hour (Fig. 1 B). After 4 hours a down regulation of PR is observed.

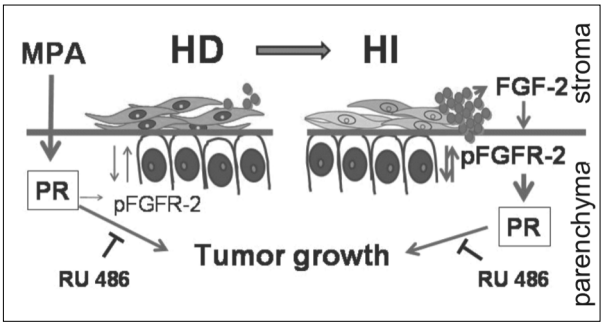

Fig 2.– Working hypothesis of HI tumor growth. Epithelial cells from HI tumors recruit activated carcinoma associated fibroblasts which secrete high levels of FGF-2 which activate FGFR-2 from the epithelial cells. This FGF-2—FGFR-2 pathway crosstalks with PR to induce cell proliferation. The blockage of FGFR-2 or PR pathways inhibits cell proliferation.

Selected studies in T47D cells

To further evaluate the role of these interactions in other breast cancer models, we used T47D human breast cancer cells that overexpress PR. Similar results regarding cell proliferation were found when CAF were incubated together with T47D cells, and as can be observed in Fig. 1 A, T47D cells gave the same response to MPA and FGF-2 as C4-HD and C4-HI. In addition, nuclear co-localization between PR and FGFR-2 was also observed (Fig. 1 C).

Conclusions

The results reported herein highlight the role of the stroma in HI tumor growth. We propose that HI epithelial cells recruit activated CAF which secrete stromal factors such as FGF2, which by binding to their cognate receptors, activate and phosphorylate PR. Novel mechanisms involving nuclear interaction between a classical transcription factor (PR) and a classical membrane receptor (FGFR-2) may activate proliferative pathways (Fig. 2).

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It is not easy to convey, unless one has experienced it, the dramatic feeling of sudden enlightenment that floods the mind when the right idea finally clinches into place.

No es fácil expresar, a menos que uno lo haya vivido, el sentimiento dramático de una luz que inunde la mente cuando la idea exacta finalmente encaja en el lugar preciso.

Francis Crick (1916-2004)

Premio Nobel, 1962