

Wound response after intraoperative radiotherapy

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Abstract: Activation of the inflammatory wound response after breast surgery has been proposed as one of the mechanisms that could stimulate breast cancer (BC) recurrence formation. This hypothesis well fits with the notion that tumors behave as “wounds that do not heal” and raised the idea that targeting the inflammatory wound response immediately after surgery could also impact on the formation of recurrences. Therefore, a clearer picture of the pathways activated by the inflammatory wound response in residual BC cells and in breast microenvironment is of primary relevance in order to identify new possible lines of therapeutic intervention. The introduction of intraoperative radiotherapy (IORT) in the clinical management of BC patients has not only allowed for treatment improvement of selected patients, but has also offered the unique opportunity to study the immediate effect of radiotherapy (RT) on human tissues, *in vivo*. One of the key unanswered questions regarding the irradiation of peri-tumoral breast tissue is whether RT could have other effects than mere cancer cell killing and how these additional effects impact on the wound response of BC patients. Here, we review the last evidences connecting breast surgery to the formation of BC recurrences and the effects of breast irradiation with IORT to the modification of the wound response. Moreover, we highlight the possibility to employ new specific peri-operative intervention that, by targeting the wound response in the appropriate window of time, administered alone or in association with IORT, may prevent BC recurrence formation.

Keywords: Wound healing; intraoperative radiotherapy (IORT); breast cancer (BC); STAT-3; p70S6K1

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Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide and the second leading cause of cancer-related death. More than 1-2 million cases are diagnosed every year, affecting 10-12% of the female population and accounting for 500,000 deaths per year (1). In the last two decades, mortality rates have generally remained stable or slightly decreased. Declines in BC mortality have been attributed to both novel treatment strategies and early detection due to the implementation of screening/prevention programs (2).

BC is not a single disease: it is instead a collection of breast diseases that are heterogeneous in terms of histology, genetic and genomic variations, therapeutic response and

patient outcomes (3). From a clinical point of view, BC can be subdivided into three major subtypes: tumors expressing estrogen receptors (ERs) and/or progesterone receptors (PRs), tumors expressing amplified form of human epidermal receptor 2 (HER2-amplified) and tumors commonly referred to as triple-negative BC (TNBC), due to lack of or low positivity for ER, PR and HER2 (4). These markers together with other clinical parameters (age, node status, tumor size, histological grade) are routinely used in the clinic to stratify patients for prognostic predictions and treatment selection. However, the complexity of BC disease is not entirely reflected by the parameters described. More recent studies have provided new ways of classification of BC patients, based on variations in their gene expression profiles that

correlated with prognosis (5-8), establishing five BC intrinsic subtypes (Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like) and a normal breast-like group.

Importantly, these subtypes have been shown to be clinically meaningful and can divide patients into groups with distinct tumor histotypes and distinct outcomes. However, up to now the expression of the ER, PR, and HER2 receptors that are routinely and easily evaluated in any pathology, are still the parameters used by oncologists to guide therapy decisions. For BC patients, several treatment options are currently available in neoadjuvant and adjuvant settings. These include hormone therapies, targeted therapies, radiotherapy (RT) and various chemotherapy regimens.

Local recurrence in early breast cancer (EBC)

The systemic use of widespread mammographic screening has contributed to a stage shift for newly diagnosed disease, increasing the percentage of EBC at diagnosis. In women with EBC all detectable cancer is restricted to the breast and, in women with node-positive disease, to the local lymph nodes. Breast conserving therapy, including primary tumor excision, axillary node dissection (determined in advance or decided following sentinel node sampling) and external RT, is considered standard of care for management of women with EBC (9). For EBC patients the appearance of local relapse (LR, defined as the reappearance of malignant disease in the ipsilateral breast) represents a common event that may influence the prognosis. Several studies have shown that the presence of local recurrence is the strongest independent predictor of distant relapse and confers three-fold to four-fold increased risk of progression (10). Importantly, it was demonstrated that local recurrence formation is causally related to distant relapse, indicating that it is a determinant and not simply an indicator of augmented risk (11). Disease relapse occurs in one out of five BC patients and represents the principal cause of BC-related deaths (12). The relative risk of distant metastases for patients developing LR in comparison with patients without LR is considerable and, in fact, patients who develop LR present a substantially worse overall survival (13-15). The importance to restrain local recurrences in BC patients has been recently highlighted in a overview that conclusively showed that treatments substantially improving local control have better effects on long-term survival, representing one life saved for every four loco-regional recurrences prevented (16).

The effects of external RT on local as well as distant recurrence formation and on long-term overall survival of BC patients were recently extensively analyzed. A recent meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) has demonstrated that local radiation treatment to either the breast after breast-conservation surgery or the chest wall after mastectomy induced an overall survival benefit at 15 years (16). In particular, RT reduces the recurrence rate by the half and the death rate by about one sixth in patients that have undergone breast conserving surgery. Results of the EBCTCG overview have thus reinforced the link between local control and mortality, emphasizing the importance of achieving the best loco-regional treatment for this kind of patients.

Local recurrence in early breast cancer (EBC) patients and the wound response

Clinical and experimental data suggest that the perturbation induced by surgery itself and the subsequent wound healing process may result in stimulation of residual disease (17-19). From a clinical point of view, 90% of local recurrences occur at or close to the same quadrant of the primary cancer, despite multifocality and multicentricity are very common in BC (20). Three-dimensional analysis of specimens from mastectomy show that 63% of breasts harbor occult cancer foci, with 80% of these situated remote from the index quadrant. However, the cancers in other quadrants of the breast appear to remain dormant for many years and have a low risk of developing clinically relevant tumors (20). The results of a randomized clinical trial comparing the effects of mastectomy with quadrantectomy showed that early relapses were more frequent in the mastectomy than in the quadrantectomy group. This difference, which disappeared later in time during follow-up, is consistent with an initial acceleration of metastatic burden after more aggressive surgery (14). These clinical observations support the idea that local disease develops from growth of residual BC cell in peritumoral tissue, in response to the inflammatory and wound-healing stimuli elicited by surgery. Experimental data in animal models show that the surgical trauma enhances the proliferation of metastatic foci, supporting the hypothesis that surgery should be considered a major perturbing factor for metastasis growth (21,22). Similarly to what observed in animal models, also in human BC some evidences highlight that surgical removal of the primary tumor may induce changes in the growth kinetics of micro-

metastasis (18).

Wound healing and cancer progression have striking similarities, including inflammation, the growth of new blood vessels (angiogenesis), the rearrangement of the molecular matrix around the cells, and changes in how cells attach to each other, leading to the definition of tumors as wounds that do not heal (23-25). Moreover, the molecular programs in normal wound healing and those in tumor progression and metastasis were found to be similar. The correlation between wound response and cancer progression was also supported by the analysis of gene expression profile of normal tissue adjacent to cancer that evidences the activation of a “wound response signature” able to promote cancer progression (26). The activation of this “wound response signature” is highly prognostic of poor survival in BC patients, demonstrating that the status of the tumor microenvironment represents an important variable for BC progression (26) and strongly suggesting the potential relevance of the wound response induced by surgery.

Studies performed in mice have shown the presence of growth stimulating factors in mouse serum after removal of the primary tumor (22) and recently the stimulatory effects of post-surgical drainage fluids harvested for 24 hours after surgery from BC patients (hereafter referred to as wound fluids, WF) have also been tested (27,28). Tagliabue *et al.* reported that WF, as well as postsurgical serum samples, induce proliferation of HER2-positive breast carcinoma cells, signifying that at the site of surgery growth factors able to induce BC cells proliferation are secreted (28). It has also been demonstrated that WF collected from EBC patients undergone breast surgery, stimulated cancer cell growth, migration and invasion (27), supporting the role of surgery as perturbing factor for recurrence formation, also in human patients.

Based on these observations, we pursued the identifications of the pathways specifically activated in BC residual cells by the wound response after surgery. Our very recent work highlighted the relevance in this context of two signaling pathways, the p70S6K and the STAT3 pathway.

p70S6K signaling and breast cancer (BC)

The PI3K/mTOR/p70S6K signaling axis is known to regulate many processes in the cells, many of which are critical for tumorigenesis, such as cell growth, proliferation, survival and metabolism (29). Briefly, the activation of PI3K (phosphatidylinositol 3-kinase) and the production of the lipid second messenger PIP3 (phosphatidylinositol

3,4,5-trisphosphate) from PIP2 trigger the recruitment and activation of Akt (protein kinase B) by the phosphatidylinositol-dependent kinases, PDK1 and PDK2 (30). One of the major downstream effectors of Akt is mTOR. Akt phosphorylates and inactivates the tuberous sclerosis complex (TSC) tumor suppressor, leading to the activation of the mammalian target of rapamycin complex 1 (mTORC1, hereafter mTOR) signaling pathway that results in the activation of p70S6K (31).

The ribosomal protein S6 kinase family comprises two homologous proteins, S6K1 and S6K2, each of which are found as two alternatively spliced isoforms (p70S6K and p85S6K, in the case of S6K1). Both S6K1 and S6K2 are downstream of mTOR pathway and exert same redundant functions. The p70 kDa isoform of S6K1 (p70S6K1) is the most studied S6 kinase, it is ubiquitously expressed and localizes predominantly to the cytoplasm (32). S6K1 plays important roles in cell growth, proliferation, and differentiation, mainly by regulating protein synthesis, cell cycle progression and metabolism (33-35).

It is extensively reported that p70S6K pathway is inappropriately activated in many cancer types, by receptor tyrosine kinases, as well as by the genetic mutation and overexpression of other key pathway components such as PI3K, Akt or loss of expression/function of negative regulators, such as the tumor suppressors PTEN and TSC1/2. The aberrant activation of this pathway plays a major role in BC and many evidences suggest that it is linked to promotion of BC cell growth and survival, resistance to chemotherapy, resistance to endocrine therapy and it is associated with poor prognosis, advanced stage and histological grade (36-38).

Recent studies have addressed the specific role of S6K1 in tumor proliferation, invasiveness, motility and angiogenesis (39,40). In particular, many data suggest the involvement of p70S6K1 also in BC onset and/or progression. p70S6K1 is encoded by the RPS6KB1 gene that is amplified in ~9% of primary breast tumors, leading to the overexpression of the protein (41-44). Moreover, RPS6KB1 amplification and overexpression are associated with poor prognosis in an unselected series of BC patients (43). Interestingly, activation of p70S6K1 (monitored by evaluation of phosphorylation status of Thr389 residue) has been found elevated by 10- to 35-folds in BC cells compared to normal primary mammary epithelial cells (45). Moreover, more than 70% of invasive breast carcinomas, have been demonstrated to possess high levels of phosphorylated p70S6K1 and, in sharp contrast, phosphorylation of the same protein was

nearly undetectable in normal mammary tissues under the same assay (45). Furthermore, overexpression of p70S6K1 protein is linked to increased risk of locoregional recurrence in node-negative EBC patients, thus suggesting a role for this kinase also as prognostic marker (46).

Role of p70S6K signaling pathway in breast cancer (BC) recurrence

Using WF drained from BC patients after surgery as a model to study the impact of the wound response in recurrence formation, Segatto *et al.* recently demonstrated that BC cells responded to WF stimulation hyper-activating p70S6K pathway and this activation positively contributes to proliferation and invasion programs of BC epithelial cells, *in vitro* (47,48). Impairment of p70S6K1 signaling slightly decreased primary breast tumor growth in nude mice and an intact p70S6K1 signaling was extremely critical for tumor initiation, as demonstrated by tumor take-rate analyses. More interestingly, impairment of p70S6K1 signaling slightly increased the tumor latency (9 versus 26 days) but, once tumors appeared, their growth rate was very similar. This observation pointed out that, in the process of tumor initiation, p70S6K1 signaling played a major role in survival rather than in proliferation of BC cells. This hypothesis was also supported by the results from pharmacological inhibition of p70S6K1 activity *in vivo* demonstrating that a specific p70S6K1 inhibitor, PF-4708671, impairs cancer cell survival in hostile condition while having minor effects, if any on the growth of established tumors (47,48).

These data strongly support the observation that p70S6K1 plays a fundamental role in the formation of LR. Using a preclinical model that mimics the clinical setting Belletti and coworkers showed that interfering with p70S6K activity had a significant impact on BC cell behavior and almost completely prevented formation of local recurrence. Importantly, a three-day schedule of peri-operative treatment with the specific PF-4708671 was sufficient to significantly prevent the appearance of relapses, demonstrating that this critical event takes place in very narrow window of the disease (47). From a molecular point of view, the crosstalk between p70S6K signaling and the Hedgehog-Gli1 pathway was found to be crucial to activate the survival response needed by BC cells to escape apoptosis in critical contexts, as recently suggested also by others in other cancer types (48-50).

Importantly, activation of p70S6K1 after surgery

takes place also in BC patients. In fact, in paired BC specimens from patients who underwent lumpectomy first and surgical widening to clear margins 1-2 weeks later, nearly 50% of patients displayed an increase of p70S6K1 activity in the second specimen respect to the first and only 8% showed a reverse trend, thus strongly supporting the hypothesis that p70S6K1 activity is increased by post-surgery stimuli (47).

Relevance of p70S6K1 in the survival of BC cells, more than in proliferation *per se*, is also supported by the work of Akar *et al.* demonstrating in a BC xenograft model that survival and engraftment of lung metastasis relies on p70S6K1 activity (51).

Inhibition of mTOR by temsirolimus should, in principle, elicit the same response of p70S6K1 inhibition in BC recurrence formation but this seemed not to be the case. Administration of temsirolimus did not reduce BC cell survival *in vitro* nor of local recurrence *in vivo*, probably because blocking the p70S6K negative feedback loop, led to paradoxical hyperactivation of AKT and up-regulation of Bcl2, which, in turn, fostered cell survival. This finding supported the notion that, under prolonged Tems treatment, isolated cells activated a survival response that is avoided when only p70S6K1 is specifically inhibited (47,48,52). These *in vitro* and preclinical data indicate that caution should be used in the administration of mTOR to BC patients since their use could lead to paradox activation of canonical survival pathways in residual BC cells.

Overall several works point to the activation of p70S6K1 as a key determinant of BC cell survival in the post-surgery microenvironment and suggest that impairing its activity could positively impact on prognosis of BC patients. Yet, specific and clinically tested p70S6K1 inhibitors will be necessary to transfer this knowledge to the human pathology.

STAT3 signaling in breast cancer (BC)

It has long been known that inflammatory conditions can initiate or promote oncogenic transformation and cancer-associated inflammation is marked by the abundant presence of specific inflammatory cells and inflammatory mediators, such as cytokines and chemokines (52). Recent evidences suggest a crucial role for signal transducer and activator of transcription (STAT) family proteins, especially STAT3, in selectively inducing and maintaining a pro-carcinogenic inflammatory microenvironment, during both initiation of malignant transformation and cancer progression (53,54).

STAT3 belongs to the STAT family of proteins, which are both signal transducers and transcription factors. At least seven members of this family have been identified, encoded by distinct genes (55).

STAT3 plays important roles in fundamental processes, including proliferation, development, differentiation, inflammation and apoptosis (54,56-58). STAT3 is activated either by growth factor receptor, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), or by non receptor tyrosine kinases, such as JAK or Src (59,60). Upon the binding of growth factors or cytokines to their cognate receptors on the cell surface, STAT3 is recruited to the cytoplasmic tail of the receptors and becomes phosphorylated on its Tyr 705. Tyrosine-phosphorylated STAT3 then dimerizes through reciprocal pTyr-SH2 domain interactions, translocates into the nucleus and binds to specific STAT-response elements in the promoters of target genes, thereby inducing the transcription of those genes essential for its physiological functions (61-64).

STAT3 regulates the transcription of several genes, involved in apoptosis, cell cycle and epithelial-mesenchymal transition (53,54,56-58). Under normal biological conditions, STAT3 activation is rapid and transient. However, it is to note that many of the downstream target genes of STAT3 encode for cytokines and growth factors, the receptors of which signal through the same STAT3, thereby providing a positive feed forward loop of autocrine and paracrine STAT3 activation (61,64-66). STAT3 has been found to be hyper-phosphorylated and constitutively activated in a large number of solid tumors and cancer cell lines, which often become addicted to its activity for continuous survival and growth (67,68).

The role of STAT3 in promoting and sustaining transformation is well documented. Conditional knockout of the STAT3 gene or inhibition of STAT3 function block v-Src induced transformation (69,70), indicating a pivotal role for STAT3 in malignant transformation. Moreover a constitutively dimerized STAT3 protein is sufficient to induce malignant transformation and tumor formation in mice (71). The exact mechanisms by which constitutively active STAT3 mediates malignant transformation and human tumor formation are still incompletely understood and continue to be investigated.

Constitutive activity of STAT3 has been observed in 35% to 60% of human breast tumors and in many BC cell lines, in which is required for continuous proliferation and resistance to apoptosis. Since no STAT3 mutations have been identified, constitutive activation of STAT3 in breast

tumors is frequently associated with the aberrant expression and/or the activity of the EGF receptor family kinases, Src or JAK (72-76). The significance of STAT3 overexpression and/or activation in BC is however not completely clear and still debated (77,78).

Recently, high interest was raised by the possibility that STAT3 played a key role in regulating the self-renewal ability of BC stem cells (79,80). Cancer stem cells or tumor initiating cells (TICs) are rare cells and many studies support their involvement in tumor recurrence, formation of metastases, as well as chemoresistance (81,82). It was demonstrated that activated JAK2/STAT3 signaling is essential for the survival of CD44+/CD24-/low BC cells (83) and it was shown to play an important role during mammosphere formation, an *in vitro* assay used to isolate and propagate BC stem-like cells (84). STAT3 was also identified by an RNAi screen, as a critical player for mammosphere formation and self-renewal of breast TIC (85,86). Despite the fact that a sizable body of evidences highlight that STAT3 is inappropriately activated in a vast percentage of breast tumors, its concrete role in BC initiation and/or progression is still very controversial (87).

Since the discovery of the association between constitutive STAT3 activation and malignant transformation, a large number of studies have been undertaken for validating STAT3 as a cancer drug target (88-90), and substantial efforts were employed into the discovery of novel STAT3 inhibitors. A large number of STAT3 inhibitors have been developed, displaying different mechanism of actions. Of these inhibitors, a few of them has been validated and show good activity in terms of the inhibition of STAT3 biological functions and the associated antitumor cell effects, as well as the inhibition of tumor growth in the mouse models of human tumors (90). Up to now, these inhibitors are mostly at the experimental stage and only a few have been tested in clinical trials, with limited success (91-93).

Role of STAT3 signaling pathway in breast cancer (BC) recurrence

WFs are extremely rich in cytokines and growth factors and represent a surrogate source of the inflammatory stimuli present in the post-surgical setting in breast microenvironment. Strong and specific activation of STAT3 is induced in BC cell lines following WF stimulation. Moreover, WF-induced STAT3 activation was far more pronounced respect to activation induced by other mitogenic stimuli, indicating a specific role of STAT3

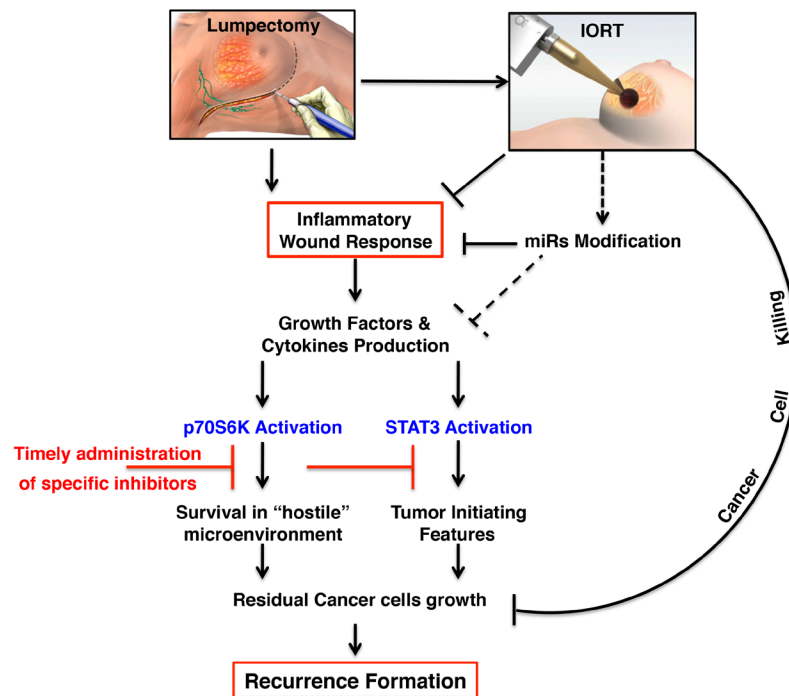


Figure 1 In BC patients after lumpectomy, a wound healing response is activated by the act of surgery, leading to local release of growth factors and cytokines. The wound response induces pro-survival and pro-tumorigenic pathways (e.g., p70S6K and STAT3 pathways) in residual tumor cells that will eventually lead to BC recurrence. Application of IORT immediately after lumpectomy on one side sterilizes the tumor bed of residual cancer cell and, on the other, impacts on the wound response by modifying the peri-tumoral microenvironment. Timely administration of inhibitors (peri-operative schedule) blocking p70S6K and STAT3 pathways, alone or in combination with IORT, may represent a valuable therapeutic approach to prevent recurrence formation in BC patients. BC, breast cancer; IORT, intraoperative radiotherapy.

signaling pathway in this context (94).

In accord with the role proposed for STAT3 in the growth maintenance and self-renewal ability of TICs, Segatto *et al.* observed that WF is able to efficiently stimulate the cancer initiating phenotypes and self-renewal potential of BC cells (94). Genetic and/or pharmacological inhibition of STAT3 completely prevented self-renewal of BC cells stimulated with WF, suggesting that the inflammatory stimuli present in the post-surgical setting in breast microenvironment mediated TIC proliferation at least in part, via STAT3 activation (94).

In agreement with these observations, STAT3 activity not only positively impacted on the initiation of breast tumorigenesis *in vivo*, but, more importantly, was detrimental in the process of recurrence formation. In a mouse model of LR, the inhibition of STAT3 activity decrease the appearance of recurrences, suggesting that STAT3 activation plays a pivotal role in the regulation of the processes that lead to the re-growth of the tumor (94).

Wound response after intraoperative radiotherapy (IORT)

TARGIT-A trial was launched in 2000 to test whether IORT might be considered an alternative to external RT (20,95). IORT delivers a high dose of radiation as one single fraction at the time of surgery, allowing the precise application of radiation to the target area around the surgical bed. The clinical outcome of TARGIT application in EBC patients was recently reported, showing that the intraoperative treatment is more effective than previously hypothesized (95). From a molecular point of view, Belletti *et al.* evaluated whether treatment with intra-operative RT may reduce local recurrence by contextually killing residual tumor cells and affecting the peri-tumor microenvironment (27). Both *in vitro* results (27) and clinical observations (95) suggest that clinical success of TARGIT may be due, at least in part, to the alteration of the microenvironment through the modulation of the wound healing response induced by intra-operative RT. In fact, WF derived from TARGIT-treated patients were defective in

activating both p70S6K and the STAT3 signaling pathways if compared with WF from patients treated only with surgery, while other pathways, such as AKT or ERK, were essentially unaffected (27).

These differences in pathway activation had functional consequences, since the stimulation of BC cell motility, invasion and growth observed with WF harvested from patients who have undergone wide local tumor excision was significantly abrogated when the WF were harvested from TARGIT treated patients (27).

A proteomic analysis on WF evaluating the levels of 174 cytokines, demonstrated that TARGIT treatment modified the levels of several factors involved in the control of cell growth and motility, such as IL-6, IL-8, HGF, UPA, Leptin and Rantes (27). Moreover, in accord with previous observation on the effects of RT on cytokines expression modification in humans and animals models (96-98), a specific increase in IL-5 and IL-4 following TARGIT was observed. This cytokine imbalance could eventually dictate a different immune response in local microenvironment, eventually amplifying the anti-tumoral effects of IORT. In line with this hypothesis, the modification of immune response by RT has been recently proposed (99,100). Moreover, it has been recently reported that application of IORT to BC patients induces a rapid and reproducible modification of microRNA expression that could eventually modify the crosstalk between residual tumor cells and the post-surgery microenvironment thus participating to the antitumor response of IORT (101).

Overall, it is thus possible that intra-operative radiotherapeutic treatments, such as TARGIT, in addition to the conventionally known tumoricidal effect exert their therapeutic effects by altering the tumor microenvironment, eventually leading to reduce recurrence formation (Figure 1).

A large body of literature exists on the possible unwanted and harmful effects of RT. These include onset of cardiac toxicity (102) and appearance of second tumors (103) and, also, the paradoxical stimulation of tumor cell growth and spreading, attributed to modifications of the local microenvironment. It is known, for instance, that RT induces a hypoxic condition, which, in turn, may stimulate tumor neoangiogenesis (104,105) and promote the production of pro-metastatic growth factors, such as TGF β (106). Whether these harmful effects are related to the dose, the modality, the timing and the type of RT delivery is largely unknown. At this regard, it is possible to speculate that the effects of RT could greatly differ if radiation is applied to a wounded tissue respect to the application from the outside,

to an already repaired breast, as it is in the case of EBRT during BC therapy. The generation of appropriate models of BC recurrence coupled with model of precise irradiation on wounded and intact breasts will be necessary to verify whether these hypotheses are true.

Discussion

The possible harmful effects of surgical wounding have been speculated for a long time and have been demonstrated in mice (13,18). Also in humans, it was demonstrated that the growth kinetics of BC micro metastasis was modified by surgery, representing a perturbing factor in the process of relapse or metastasis development (19,26). Moreover, experimental and clinical observations suggest that the extent of surgery may represent a variable able to enhance tumor burden (14,17-19,107).

So, surgery and the consequent inflammatory response caused by wounding could represent factors that favor the proliferation of "residual" tumor cells. The communication between microenvironment and tumor cells plays an important role in this context. At initial stages of tumor development, the microenvironment surrounding the tumor should provide tumor-suppressive signals; however, once tissue homeostasis is lost, the altered microenvironment can itself become a potent tumor promoter, as widely demonstrated in literature (108). The process of wound healing can induce changes in the microenvironment, such that a shift the balance between tumor-suppressive and tumor-promoting signals occurs. The combination of post surgery inflammation with wound healing-induced growth factor production can breach the barrier, resulting in promotion of the growth of residual cells into a tumor.

Accordingly, wound axillary fluids harvested from BC patients have been proved to stimulate Her2-positive mammary carcinoma cell growth and this effect is only partially abrogated by impairing Her2 signal transduction (28). Our work, formally demonstrating that WF harvested from BC patients who have undergone wide local tumor excision strongly stimulate BC cell growth, motility and invasion, also shows for the first time that one single application of IORT with TARGIT is sufficient to significantly abrogate the stimulatory effects of surgical WF on cancer cells *in vitro*. These findings strongly support the idea that TARGIT may confer more benefits than those expected merely from the tumoricidal effect of RT (27). In support to this hypothesis the recent results of randomized trial TARGIT-A confirmed non-inferiority clinical outcomes

respect EBRT, only when TARGIT was delivered concurrently with lumpectomy (prepathology stratum) but not when it was delivered at a later time, through a second surgical procedure (postpathology stratum) (95). Several factors might have played a part in achieving the low recurrence rates that was identified in the stratum randomized to receive TARGIT concurrently to lumpectomy. The immediate delivery of radiation to the wounded tissue appears to be essential to achieve the beneficial effects on the tumor microenvironment, suggesting that the timing of treatment is an important variable that can determine different clinical response (95), in line with the findings reporting that TARGIT delivery significantly modifies the protein expression profile of the WF (27), the activation of signaling pathways in BC cells and also microRNA transcription and secretion (101).

The act of surgery leads to a profound modification of the local microenvironment. In that context, reactive microenvironment is able to sustain the survival and, eventually, the re-growth of residual cancer cells through the secretion of inflammatory cytokines and growth factors. Altogether, many results allow to suggest that the signaling pathways that strongly influence the response of residual BC cells are mainly two. The activation of p70S6K1 prevalently fosters the survival of these cells, while the activation of STAT3 supports the self-renewal ability of breast TICs. We can hypothesize that these pathways cooperate in the post-surgical setting to allow the survival and re-growth of residual cells, eventually leading to the formation of BC recurrence.

Taken together, the findings presented in this review of the literature provide a biological rationale for the use of molecularly targeted agents to compensate the harmful consequences of surgery. It is well recognized that improved clinical efficacy of RT represents a substantial progress in clinical practice and patient outcomes (109). Thus, the use of peri-operative targeted treatments in combination with IORT could improve the clinical response in EBC patients through the “sterilization” of microenvironment and the subsequent inhibition of the pathway mainly involved in recurrence formation, such as p70S6K and STAT3. We propose that this combination treatment, coupled with the correct timing of administration, should be soon tested to improve patient response, particularly in those BC subtypes in which response to standard therapies is currently low.

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