Stimulation of Breast Cancer Cell Lines by Post-surgical Drainage Fluids

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Abstract. *Background/Aim: Surgery, which remains a conventional treatment of breast tumors, may induce the secretion of growth factors that support angiogenesis and wound healing. These factors are suspected to trigger carcinoma cell division and promote tumor relapse. We addressed this question by culturing breast cancer cell lines in the presence of wound fluid harvested after surgery. Materials and Methods: Wound fluids were collected from patients who underwent either breast reconstruction, tumor resection, or tumor resection after neoadjuvant chemotherapy. MCF-7 (estrogen receptor (ER)$^+$/progesterone receptor (PgR)$^+$, HCC1937 (ER/PgR$^-$, human epidermal growth factor receptor/neuralized (HER2/neu)$^-$) and MCF-10A (used as a negative control) cell lines were grown in culture media supplemented with wound fluids. Results: Wound fluids drained during the three categories of procedures significantly stimulated the proliferation of MCF-7 and HCC1937 cells in a similar manner. Conclusion: This stimulatory effect on tumor cell proliferation could be attenuated by therapeutic targeting against growth factors and inflammation processes in order to avoid tumor relapse.*

Surgery, associated or not with adjuvant therapy, remains the conventional treatment for localized breast cancer. However, a high proportion of locoregional relapse in the primary tumor bed is still observed within the two to three years after treatment (1, 2). Both experimental and clinical evidence suggests that surgery modifies the tumor microenvironment: the wound repair process that takes place at the site of the surgical lesion is supported by the production of various angiogenic and cell growth factors, as well as chemokines and cytokines. For instance, basic fibroblast growth factor, vascular endothelial growth factor, epidermal growth factor (EGF), platelet-derived growth factor and transforming growth factor-beta are secreted in wound fluids (WF) during the tissue repair process (3-5). Surgical wounding has, therefore, been reported to induce tumor progression and locoregional relapse by stimulating the proliferation of remaining cancer cells (6-10).

In this study, we addressed the ability of three categories of WF to stimulate the proliferation of breast cancer cell lines. WF were harvested from patients who underwent either tumor surgery, induction chemotherapy followed by tumor surgery, or tumor surgery followed by breast reconstruction. These WF were used to supplement cell media to grow the MCF-7 (estrogen receptor (ER)$^+$/progesterone receptor (PgR)$^+$) and the HCC1937 (ER$^-$/PgR$^-$, human epidermal growth factor receptor/ neuralized (HER2/neu); triple-negative) cell lines.

Materials and Methods

Sample collection. Drainage WF were harvested from 10 patients who underwent breast reconstruction, 10 patients who underwent tumor resection and 10 patients who underwent tumor resection after neoadjuvant chemotherapy. WF were collected for 24 h or 48 h after surgery. Only WF without blood contamination were selected. WF were collected with the usual surgical suction drain. WF collected for 24 h or 48 h, were centrifuged, sterile filtered, and stored at −80˚C.

Cell lines. Experiments were performed by using the following breast cancer cell lines: MCF-7 (ER/PgR+) (a kind gift of P. Becuwe, Sigreto, Nancy, France), HCC1937 (ER/PgR$^-$, HER2/neu$^-$ (a kind gift of P. Bischoff, EA 3430, University of Strasbourg, Strasbourg, France) and the MCF-10A immortalized non-transformed epithelial cell line derived from human fibrocystic mammary tissue that was used as a negative control (also a kind gift of P. Becuwe). MCF-7 and HCC1937 cells were grown either in complete medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum according to standard procedures, as a positive control; or in DMEM supplemented with 5% WF. MCF-10A cells were grown in either complete medium:

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DMEM-F12 supplemented with 5% horse serum, 0.02 mg/ml of EGF, 500 μg/ml of hydrocortisone, 100 μg/ml of cholera toxin and 0.01 mg/ml of insulin according to standard procedures, or in DMEM-F12 supplemented with 5% WF.

Sulforhodamine B colorimetric assay. The proliferation of MCF-7 and HCC1937 cells was evaluated by the sulforhodamine B colorimetric assay (Sigma, Saint-Quentin-Fallavier, France). Five thousand cells per well were seeded into 96-well plates. The cells were serum-starved for 48 h and were then incubated in the presence of medium with 5% of WF. Incubation with a 5% serum solution or a 0.5% serum solution were used as positive and negative controls, respectively. After six days, cell monolayers were fixed with 10% trichloroacetic acid (Sigma, Saint-Quentin-Fallavier, France) and stained with 0.4% sulforhodamine B solution for 30 min. Excess of dye was then removed by washing repeatedly with 1% acetic acid solution. The protein-bound dye was dissolved in 10 mmol/l Tris base solution for optical density determination at 550 nm, and absorbance was measured using a microplate reader (Synergy HT, Biotek, Colmar, France). All the values were normalized with respect to the absorbance of the positive control.

Trypan blue cell counting. Due to the formation of a precipitate in the growth medium during regular cell culture that interfered with sulforhodamine B colorimetric assay, the proliferation of MCF-10A cells was evaluated by trypan blue cell counting. Thirty thousand cells per well were seeded into 24-well plates. The cells were serum-starved for 48 h and were incubated in the presence of medium with 5% of WF. Incubation with a 5% serum solution or a 0.5% serum solution were used as positive and negative controls, respectively. After five days, cell monolayers were trypsinized and the total number of cell was determined by blue trypan cell counting using a Malassez C-chip. All the values were normalized with respect to the cell number in the positive control.

Statistical methods. Data were analyzed using a Kruskal-Wallis one-way analysis of the variance. Differences were considered significant when $p < 0.05$. The computer program PRISM (version 4; GraphPad, Inc., La Jolla, CA, USA) was used.

Results and Discussion

Mammary cell lines were cultured with media supplemented with draining WF from patients who underwent either tumor surgery, or induction chemotherapy followed by tumor surgery, or tumor surgery followed by breast reconstruction, and the mean relative cell growth is presented in Figure 1A. MCF-10A immortalized, non-tumorigenic cell line, used as a negative control, did not show any proliferative behavior when cultured in the presence of WF. MCF-7 and HCC 1937 cells grown in non-supplemented medium showed a similar growth rate. However, the proliferation of both MCF-7 and HCC 1937 cells was stimulated when grown in the presence of WF. The proliferation index of MCF-7 and HCC 1937 grown in the presence of WF was found to be similar to the one obtained when these cell lines are grown using standard conditions (growth-medium supplemented with fetal bovine serum). Thus, it is likely that this proliferative behavior is induced by growth factors know to be secreted in the extracellular milieu during the wound healing process. In particular, basic fibroblast growth factors, vascular endothelium growth factor, and transforming growth factor-beta have been reported to play a crucial role in the activation of cell proliferation of breast cancer cells (10, 11).

We further analyzed these results by stratifying the observed growth according to the origin of WF. WF from patients who underwent tumor surgery, preoperative induction chemotherapy, or tumor surgery followed by breast reconstruction induced MCF-7 cell proliferation to similar extents (Figure 1B). Conversely, the growth measured for the HCC1937 cell line grown in the presence of WF drained from wounds of the three categories of patients was found to be increased. Interestingly, the proliferation index obtained by culturing HCC 1937 cells was significantly higher than that observed in MCF-7 cells, suggesting that triple-negative cell lines are more sensitive to stimulation by WF. A statistically higher growth index was recorded when HCC1937 cells were cultured with WF harvested from patients who underwent breast reconstruction, compared to patients who underwent tumor resection or breast reconstruction.

These data confirm that cancer cell proliferation can be stimulated by WF collected from patients who undergo surgery for breast tumor. Interestingly, the potential of WF to stimulate cell proliferation was not found to vary according to the therapies that were proposed to the patient. In particular, induction chemotherapy provided before breast tumor surgery seems to increase the growth index of triple-negative breast cancer cell lines grown in the presence of WF from these patients. This suggests that the systemic effects of chemotherapy might influence the levels of soluble growth factors and angiogenic factors the site of the surgical wound of these tumors. The fact that this effect is observed in triple-negative cell lines only suggests that these tumors and/or their microenvironment might respond differently to these factors.

Proliferative effects of WF on breast cancer cell cultures were first reported by Tagliabue and collaborators, who showed that growth of HER2^{+} cells lines was more strongly stimulated than was that of non HER2-expressing cell lines (12). More recently, Belleti and colleagues showed that WF stimulated the S-phase entry of breast carcinoma cells, but also promoted cell migration and invasion (8). We similarly observed that WF are able to induce breast carcinoma cell proliferation: our results are therefore consistent with these previous reports.

WF are therefore suspected to be responsible for the proliferation of cancer cells that are not eliminated after surgery, and to be able to recruit distant cancer cells to the primary tumor bed. These observations provide an explanation for local recurrence that is observed at the scar site two to three years after breast tumor surgery.
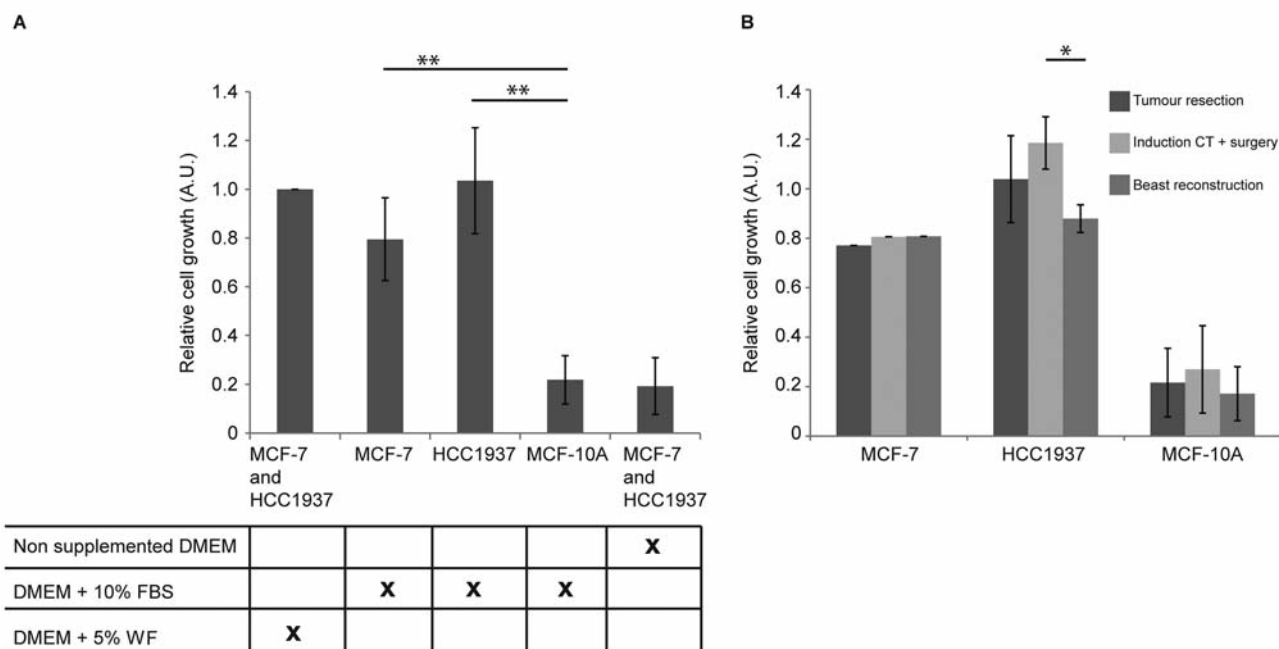
Figure 1. *A: Relative cell growth of MCF-7 and HCC1937 breast carcinoma cell lines, and of MCF-10A non-transformed breast cell line (used as a negative control), as determined by sulforhodamine B colorimetric assay and trypan blue cell counting, respectively. Cell lines were grown in growth medium supplemented with 5% wound fluids (WF) harvested from patients with breast cancer after surgery. MCF-7 and HCC1937 grown in non-supplemented media, or in media supplemented with standard fetal bovine serum (FBS) were used as negative and positive controls, respectively. WF were found to induce MCF-7 and HCC1937 cell proliferation, compared to negative controls. Results were statistically significant (Kruskal–Wallis; p<0.01**). B: Analysis of the relative cell growth of MCF-7, HCC1937 and MCF-10A cell lines grown in media supplemented with WF harvested from breast cancer patients who underwent tumor resection, induction chemotherapy (CT) or breast reconstruction. HCC1937 cell lines showed a higher proliferation index as compared to MCF-7 cells, in all surgical conditions (Kruskal–Wallis; *p<0.05; **p<0.01). HCC1937 cells grown in the presence of WF from patient who underwent preoperative CT displayed a higher proliferation index (Kruskal–Wallis; *p<0.05; **p<0.01).*

Several therapeutic alternatives could be envisioned in order to reduce the effect of angiogenic and growth factors known to be secreted in WF. Incubation of the HER2-expressing cells MDA-M-453 with the antibody to HER2 trastuzumab prior to culturing the cells with WF was shown to reduce the induced cell proliferation (12). Interestingly, WF drained from a patient surgically treated for a head and neck squamous cell carcinoma was shown to contain EGF and to promote the growth of EGF receptor expression in head and neck squamous cell carcinoma cell lines. This effect was impaired by using the antibody against EGF receptor cetuximab or the tyrosine kinase inhibitors gefitinib and lapatinib (known to target EGF receptor, HER2 and HER3) (13). Consistently, EGF and tumor growth factor beta, two EGF receptor ligands were found in WF from patients with head and neck squamous cell carcinoma. Using a mouse model of microscopic residual disease, the authors of this study showed that cetuximab inhibits tumor growth and prolongs overall and disease-free survival of mice (14). The induction of cell migratory and invasive properties, and of cell proliferation to a lesser extent, was impaired when breast carcinoma cells were grown in media supplemented with WF from patients who received targeted intraoperative radiotherapy, suggesting that irradiation modified the tumor microenvironment and the composition of WF (8). Implantation of the SCC VII squamous cell carcinoma cell line in surgical wounds created in syngenic mice models resulted in increased accelerated tumor growth. This growth was impaired when mice were treated with nonsteroïdal anti-inflammatory drugs such as Celecoxib (anti-cyclooxygenase- 2 agent) and indomethacin (inhibitor of prostaglandin production) (15).

In conclusion, we believe it is reasonable to propose that providing breast cancer patients with perioperative treatments that impact the primary tumor bed or modify WF composition might achieve improved locoregional control of the disease. Thus the stimulatory effect of injury on tumor cell proliferation can possibly be attenuated by therapeutic targeting of growth factors and inflammation.

Acknowledgements

References

1 Demicheli R, Terenziani M, Valagussa P, Moliterni A, Zambetti M and Bonadonna G: Local recurrences following mastectomy: support for the concept of tumor dormancy. J Natl Cancer Inst *86(1)*: 45-48, 1994.

2 Fisher B, Anderson S, Bryant J, Margolese RG, Deutsch M, Fisher ER, Jeong JH and Wolmark N: Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. N Engl J Med *347(16)*: 1233-1241, 2002.

3 Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG and Werner S: Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem *270(21)*: 12607-12613, 1995.

4 Lynch SE: Interactions of growth factors in tissue repair. Prog Clin Biol Res *365*: 341-357, 1991.

5 Moulin V: Growth factors in skin wound healing. Eur J Cell Biol *68(1)*: 1-7, 1995.

6 Abramovitch R, Marikovsky M, Meir G and Neeman M: Stimulation of tumour growth by wound-derived growth factors. Br J Cancer *79(9-10)*: 1392-1398, 1999.

7 Baker DG, Masterson TM, Pace R, Constable WC, Wanebo H: The influence of the surgical wound on local tumor recurrence. Surgery *106(3)*: 525-532, 1989.

8 Belletti B, Vaidya JS, D'Andrea S, Entschladen F, Roncadin M, Lovat F, Berton S, Perin T, Candiani E, Reccanello S, Veronesi A, Canzonieri V, Trovò MG, Zaenker KS, Colombatti A, Baldassarre G and Massarut S: Targeted intraoperative radiotherapy impairs the stimulation of breast cancer cell proliferation and invasion caused by surgical wounding. Clin Cancer Res *14(5)*: 1325-1332, 2008.

9 Bogden AE, Moreau JP and Eden PA: Proliferative response of human and animal tumours to surgical wounding of normal tissues: onset, duration and inhibition. Br J Cancer *75(7)*: 1021-1027, 1997.

10 Hofer SO, Shrayer D, Reichner JS, Hoekstra HJ and Wanebo HJ: Wound-induced tumor progression: a probable role in recurrence after tumor resection. Arch Surg *133(4)*: 383-389, 1998.

11 Curigliano G, Petit JY, Bertolini F, Colleoni M, Peruzzotti G, de Braud F, Gandini S, Giraldo A, Martella S, Orlando L, Munzone E, Pietri E, Luini A and Goldhirsch A: Systemic effects of surgery: quantitative analysis of circulating basic fibroblast growth factor (bFGF), Vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-beta) in patients with breast cancer who underwent limited or extended surgery. Breast Cancer Res Treat *93(1)*: 35-40, 2005.

12 Tagliabue E, Agresti R, Carcangiu ML, Ghirelli C, Morelli D, Campiglio M, Martel M, Giovanazzi R, Greco M, Balsari A and Ménard S: Role of HER2 in wound-induced breast carcinoma proliferation. Lancet *362(9383)*: 527-533, 2003.

13 Licitra L, Perrone F, Tamborini E, Bertola L, Ghirelli C, Negri T, Orsenigo M, Filipazzi P, Pastore E, Pompilio M, Bossi P, Locati LD, Cantu' G, Scaramellini G, Pilotti S and Tagliabue E: Role of EGFR family receptors in proliferation of squamous carcinoma cells induced by wound healing fluids of head and neck cancer patients. Ann Oncol *22(8)*: 1886-1893, 2011.

14 Sano D, Gule MK, Rosenthal DI, Bell D, Yates J, El-Naggar AK and Myers JN: Early postoperative epidermal growth factor receptor inhibition: safety and effectiveness in inhibiting microscopic residual of oral squamous cell carcinoma *in vivo*. Head Neck *35(3)*: 321-328, 2013.

15 Roh JL, MW Sung and Kim KH: Suppression of accelerated tumor growth in surgical wounds by celecoxib and indomethacin. Head Neck *27(4)*: 326-332, 2005.