Original paper

Lack of healing reaction in the cancer wound

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Surgical wounds in cancer patients have a relatively high dehiscence rate. Although cancer resections are performed so as to include macroscopically non-involved tissues, some cancer cells can be present in the line of transection or surrounding tissues (R1 and R2 resections). The local healing process may facilitate proliferation of these localized cancer cells, and the high cytokine concentration within the healing wound may also attract cancer cells from distant sites to migrate into the wound area. The question arises how the tumor environment influences the wound healing process.

The aim of the study was to monitor and compare, using immunohistochemical methods, the healing process of an incision wound performed through a metastatic liver tumor of colon cancer with the healing of a normal liver incision wound. The experiments were carried out on a CC531 colon cancer rat model.

We observed impaired healing of cancer wounds at all stages of wound healing. Significantly fewer mononuclear cells infiltrated the cancer than the normal liver wounds. There were no significant differences in the phenotypes of infiltrating mononuclear cells. BrdU incorporation showed rapid proliferation of cancer but not infiltrating cells or fibroblasts in the cancer wounds. We observed no connective tissue formation and poor collagen deposition in cancer wounds. Additionally, cancer wounds were significantly deprived of newly formed vessels.

We confirmed that the impaired migration and proliferation of inflammatory cells in cancer wounds and poor scar tissue formation contribute to impaired healing of cancer 'contaminated' wounds.

Key words: cancer wound, wound healing, liver metastases.

Introduction

Surgical excision of cancer tissue is aimed at removal of the bulk of the tumor mass. However, even if tissue is transacted at a large distance from the tumor edge, it is possible that individual tumor cells may be present in the presumed non-cancerous tissues (R1 resections). In certain clinical circumstances palliative excisions are performed close to the tumor mass or even without macroscopically clean margins (R2 operations). In such cases residual tumor cells become involved in the wound healing process. Tumor cells proliferate with a net mass increase, whereas the neighboring normal tissue undergoes retraction and scar formation. The wound environment may accelerate tumor growth and subsequently lead to wound dehiscence [1]. The question arises how the cancer environment influences all stages of the healing process.

The normal wound healing process can be divided into three stages: 1) inflammatory, 2) proliferative, and 3) repair and remodeling. The inflammatory stage is initiated by blood coagulation and platelet degranulation. In response to released chemotactic factors, monocytes enter the wound and mature into wound macrophages. Wound macrophages phagocytose wound debris and, in conjunction with infiltrating lymphocytes, release growth factors, which induce migration and proliferation of fibroblasts, epithelial cells, and endothelial cells during the proliferative phase of healing. At the end of the proliferative phase, fibroblasts produce collagen, elastin, proteoglycans and other extracellular matrix (ECM) components, resulting in scar tissue formation [2, 3]. Remodeling and repair of scar tissue is controlled by the action of metalloproteinases secreted by fibroblasts and down-regulated by production of tissue inhibitors of matrix metalloproteinases (TIMPs). It has been shown that interactions of tumor cells with normal fibroblasts enhance the invasive and metastatic potential of the tumor cells [4]. A growing number of studies demonstrate a positive correlation between angiogenesis, carcinoma-associated fibroblasts, inflammatory infiltrating cells and poor outcome, thereby emphasizing the clinical relevance of the tumor microenvironment to aggressive tumor progression [5]. Also, a number of reports have confirmed observations that inflammation may be an important cofactor of tumorigenesis in sites of chronic irritation, persistent infection and previously wounded tissue [6]. Surgery represents an event that acutely causes both inflammation and a wound healing response, strongly suggesting that it may represent a perturbing factor in the process of local recurrence or metastasis development in humans [7]. Macrophages play a role in tumor growth in conjunction with lymphocytes, by synthesizing and secreting epidermal growth factor (EGF), basic fibroblast growth factor, and transforming growth factor (TGF) α and β along with other chemokines released during wound healing and inflammation, including tumor necrosis factor α (TNF-α), interleukin 6, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [8-11]. Tumor growth results in disruption of the normal tissue architecture, and induces a wound-healing response, similar to that found in the normal healing wound. Because of these similarities, tumors are often described as 'wounds that do not heal' [12]. Two questions arise: Does the healing process in 'cancer contaminated' tissue proceed in a similar way as in the healthy tissue? And does the healing process stimulate proliferation of individual tumor cells present in the wound? Even though the mechanical dissemination of cancer cells during surgery has been discussed by the medical literature since the end of the 19th century, and was extensively studied in the 1960s, the literature on the cellular events occurring in healing cancer wounds is sparse and inconsistent [13-15]. A few studies showed correlations between events occurring during wound healing and the processes of cancer spread; however, they did not answer the basic question of what the effect of the tumor environment on wound healing is [16]. In order to address this question, a comparison of wound healing of normal and cancer-contaminated tissues is required. In our previous study we documented that presence of cancer cells in the gut anastomotic wound may hamper healing and results in dehiscence [17]. The aim of this study was to follow with immunohistochemical methods the healing of liver cancer wounds and compare it with normal liver wounds. Recruitment and phenotype of host infiltrating immune cells, proliferation of tumor cells and collagenous scar formation were evaluated in both types of wounds.

Material and methods

Animals

We used male WAG/Rij rats (250 to 300 g body weight; 8 to 9 weeks old), bred and maintained in our own facility. Rats were maintained in standard conditions, and received rodent laboratory chow and water *ad libitum*. All experimental animals were treated with accordance to guidelines of Ethical Commission of the Polish Academy of Science.

CC531 cancer cells

CC531 is a moderately differentiated and weakly immunogenic adenocarcinoma of the colon which is induced by 1,2-dimethylhydrazine-induced and is syngenic to WAG/Rij rats. CC531 cells (kindly provided by Dr P Kuppen, Leiden University Medical Centre, the Netherlands) were cultured in RPMI medium supplemented with 10% FCS, penicillin, streptomycin, and Fungizone (all from Gibco, Breda, the Netherlands), and ceftriaxone (Polfarma, Warsaw, Poland). The cultures were maintained in plastic tissue culture flasks, and incubated in 5% CO_2 at 37°C in a humidified incubator. Tumor cells were harvested from sub-confluent cultures (80 to 90% confluence) by brief (10 minutes) exposure to trypsin (Gibco) diluted 1 : 10 in PBS without Ca^{2+} or Mg^{2+} (Gibco). Cells were suspended in PBS without Ca^{2+} and Mg^{2+} supplemented with 10% FCS, and centrifuged at 400 g for 10 minutes, then resuspended in serum-free PBS without Ca^{2+} or Mg^{2+}, and centrifuged as before. Cell viability was determined by the Trypan blue exclusion method, and was always greater than 90% [18, 19].

Inoculation of CC531 cells

Rats (n = 24) were anesthetized with ether. A 2 cm long mid-line incision was made in the abdominal

wall. A suspension of 2×10^6 CC531 cells in 0.5 ml 0.9% NaCl was prepared for each animal, and injected into the portal vein. Liver metastatic-type tumors developed 6 weeks after CC531 inoculation. For the study, we used a homogeneous group of 18 rats with at least four metastatic tumors, 4 mm in size, to the liver. Six rats that did not match the study criteria were euthanized by decapitation.

Tumor and liver incision wounds

Six weeks after inoculation of tumor cells, we performed tumor and liver incisions in two groups of rats. Group 1 consisted of 18 rats with CC531 colon cancer metastatic tumors to the liver, while group 2 (the control group) consisted of 18 normal healthy rats.

In the first group (group 1) of 18 tumor rats, a 4 mm deep incision of the tumor foci was performed. In the second group (group 2) of 18 normal rats, a 4 mm deep incision of the normal liver tissue was made.

In both groups hemostatic surgical suturing of tumor and liver wounds was performed. On the 3rd, 7th and 14th day of healing, liver fragments comprising the tumor and the normal liver wounds in the size of $5 \times 5 \times 5$ mm were harvested and snap frozen in dry ice cold acetone, then stored at –70°C until further use. Characteristics of experimental groups are presented in Table I.

Bromodeoxyuridine administration

Six rats were randomly chosen from both the cancer and control groups. Intraperitoneal injection of bromodeoxyuridine (BrdU) 10 mg was administered daily to each rat on days 1 to 3 during the observation period.

Immunohistochemistry of cancer and liver wounds

On days 3, 7, and 14, samples of tumor and normal liver wounds were taken. Samples were cut on a cryostat into sections 5 μm thick, which were mounted onto polylysine-treated slides. Cryosections were fixed in alcohol for hematoxylin and eosin and for trichrome staining. For immunohistochemical staining, cryosections were fixed in cold acetone for 10 minutes, then air-dried, and incubated with goat serum (diluted 1 : 1 in Tris-buffered saline) for 20 minutes, followed by incubation for 30 minutes with primary mouse monoclonal antibodies against OX6 (for major histocompatibility complex class II; MHC II), ED1 (rat monocytes and macrophages), W3/13 (leucocytes), HIS52 (vascular endothelium), and BrdU (proliferating cells) (all from Serotec, Oxford, UK) and anti-CC531 (kindly provided by Dr P Kuppen as before). The specificity of immunostaining was confirmed by incubation of some sections without primary antibody. The antibody reactions were visualized using the LSAB-2 Alkaline Phosphatase Kit (Dako, Glostrup, Denmark), in accordance with the manufacturer's instructions. The cell subpopulations infiltrating the wound site were counted in five microscopic areas (magnification 400×) of tumor and normal liver wounds using light microscopy with Microimage software (Olympus, Japan).

Blood vessels in the wound were counted in five microscopic areas (magnification 200×) as the number of vessels per field, and the result was expressed on a semi-quantitative scale: +, 0 to 1 vessel/field; ++, 2 to 5 vessels/field; +++, 6 or more vessels/field. Identification of vessels was achieved using the method specified by Weidner for blood vessel counts: any stained endothelial cell or cell cluster separated from another microvessel structure was considered a countable microvessel [20]. Staining with the anti-CC531 antibody allowed counting of the number of individual tumor cells implanted into the cancer liver wound. Counting was performed in five microscopic fields (×400 magnification) using light microscopy with Microimage software (Olympus, Japan), and results were expressed on a semi-quantitative scale: +, 0 to 5 cells/field; ++, 6 to 10 cells/field; +++, 11 or more cells/field.

The population of BrdU-positive cells was divided into mononuclear infiltrating cells and CC531 cells. The latter were recognized by their large, irregular shape. Both populations were counted in five microscopic fields (magnification 400×) using light microscopy with Microimage software (Olympus, Japan), and results were expressed on a semi-quantita-

Table I. Study groups: Group 1: Incision of the tumor in the liver, Group 2: Incision of the normal liver tissue. Incisions were performed on day 1 of the study, bromodeoxyuridine was administered on days 1, 2 and 3 of the study, and samples were collected on days 3, 7 and 14

STUDY GROUPS	BROMODEOXYURIDINE 10 MG INJECTION	INCISION SITE	RELAPAROTOMY, SAMPLE COLLECTION.
Group 1. Tumor bearing rats (n = 18)	injection day: 1, 2, 3 (n = 6)	liver tumor, day 1	Day: 3, 7, 14
Group 2. Normal rats (n = 18)	injection day: 1, 2, 3 (n = 6)	normal liver, day 1	Day: 3, 7, 14

tive scale: +, 0 to 5 cells/field; ++, 6 to 10 cells/field; +++, 11 or more cells/field.

Deposition of collagen in trichrome-stained specimens was estimated by measuring the thickness of the blue-stained collagen bundles in the section using light microscopy with Microimage software (Olympus, Japan), expressed on a semi-quantitative scale: +, 2 μm; ++, 4 μm; +++, 6 μm. The slides were reviewed independently by three observers (WLO, MS, and MG). In the event of discrepancies between observers, the slides were reviewed once again, and the results were agreed upon by consensus.

Statistics

Results are presented as cells/field (mean ± SD). For statistical analysis the nonparametric Wilcoxon's rank sum test and *t*-test were used. $P < 0.05$ was considered significant.

Results

Mononuclear infiltrates of cancer versus normal liver wounds.

There was a significant difference in the mean count of mononuclear cells per microscopic field infiltrating cancer compared to normal liver wounds (Fig. 1A, B). Evidently fewer monocytes/macrophages and lymphocytes were seen in the cancer than in the normal liver tissue wounds, although no differences in phenotypes of cells infiltrating cancer and normal liver wounds were observed (Fig. 2A, B). Numbers and phenotypes of wound-infiltrating cells are presented in Table II.

Blood vessels

Blood vessels were observed on normal liver wound specimens on the 7th and 14th day of healing, whereas cancer wounds in the liver were almost com-

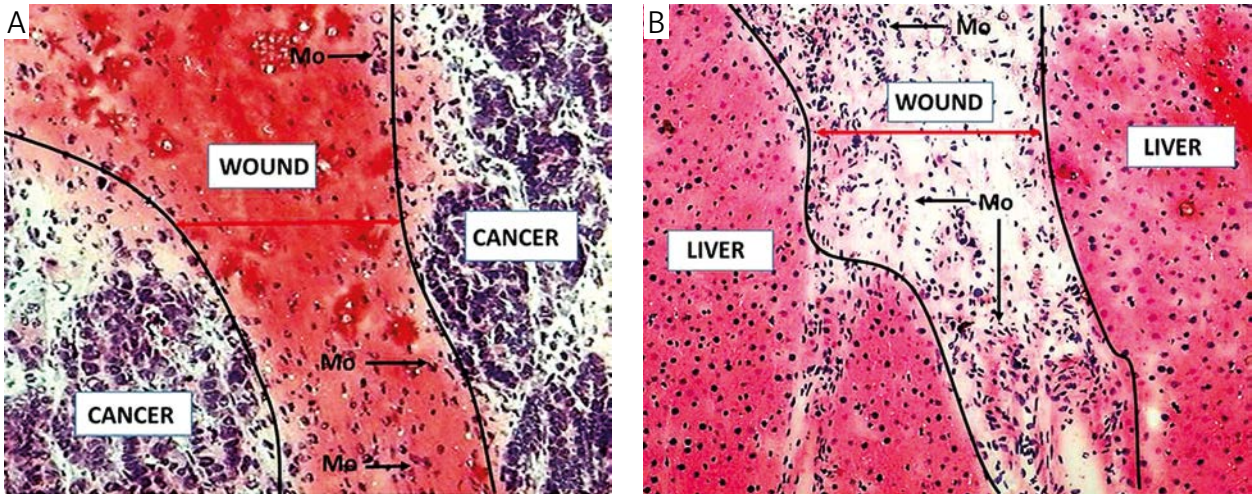

Fig. 1. A) Cancer wound. Healing day 7. HE staining. Magnification 200×. **B)** Normal liver wound. Healing day 7. HE staining. Magnification 200×. Mo – infiltrating mononuclear cells. Abundant mononuclear infiltration of the wound site *Mo – infiltrating mononuclear cells. Weak mononuclear infiltration of the wound site.*

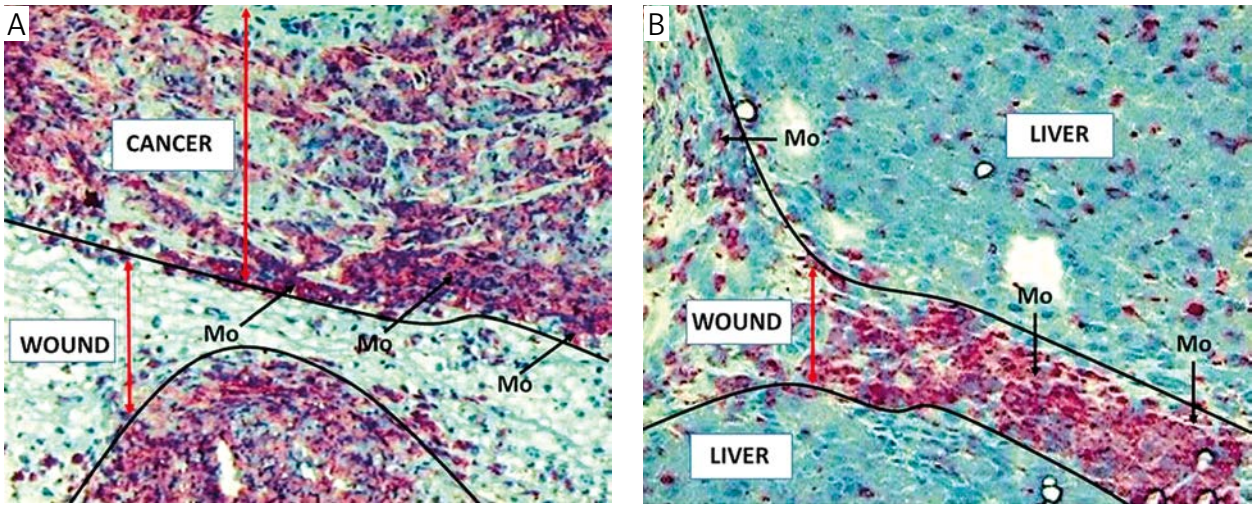

Fig. 2. A) Cancer wound. Healing day 7. Staining with CD14 Ab for monocytes/macrophages. Magnification 200×. **B)** Normal liver wound. Healing day 7. Staining with CD14 Ab for monocytes/macrophages. Magnification 200×

Table II. Mononuclear infiltrates of cancer liver wound versus normal liver wound. Mean count of mononuclear cells per microscopic field (magnification 400×) infiltrating normal and cancer wounds. Cumulated values from days 3, 7 and 14 (± SD) (n = 6, *p < 0.05)

Cell type	Cancer wound in the liver	Statistical significance	Normal liver wound
ED 1 (CD14)	12.90 ±5.46	*	36.88 ±6.95
OX6 (MHCII)	8.43 ±3.25	*	19.03 ±7.20
W3/13 (CD 3)	2.40 ±1.19	*	11.76 ±5.18

pletely deprived of vessels. Individual vessels in the cancer wounds were encountered on slides obtained on the 14th day of healing. The median blood vessel count per microscopic field in the cancer wound was significantly ($p < 0.05$) lower than in the normal liver wound (0.58 ±0.67 versus 7.50 ±5.22, respectively). The blood vessels on the 7th day of healing of the normal liver wound were located at the border between liver tissue and the wound. On the 14th day of healing vessels were more abundant and spread to the centre of the wound (Fig. 3).

BrdU incorporation

In the normal liver wounds, BrdU incorporation showed a significantly higher proliferation rate ($P < 0.05$) of mononuclear cells infiltrating the wound site compared to the liver cancer wounds: 10.75 ±3.13 versus 3.80 ±1.97 cells per field respectively. Additionally, staining for BrdU incorporation showed rapid proliferation of cancer cells on the edge of the cancer wounds (Fig. 4A, B).

Connective tissue formation

There was no connective tissue formation and poor collagen deposition in cancer wounds compared to normal liver wounds (Fig. 5A, B).

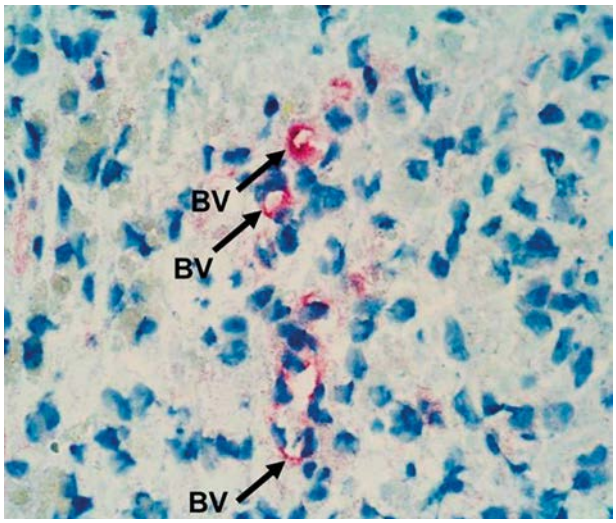

Fig. 3. Normal liver wound. Healing day 7. Staining with HIS 52 Ab for blood vessel endothelial cells. Magnification 400×

Distribution of CC531 cells

Staining for CC531 cells revealed presence of single cancer cells in the liver cancer wound. These cells were located at the border between the wound and cancer tissue, but we did not observe migration of CC531 cells into the central area of the cancer wound (Fig. 6).

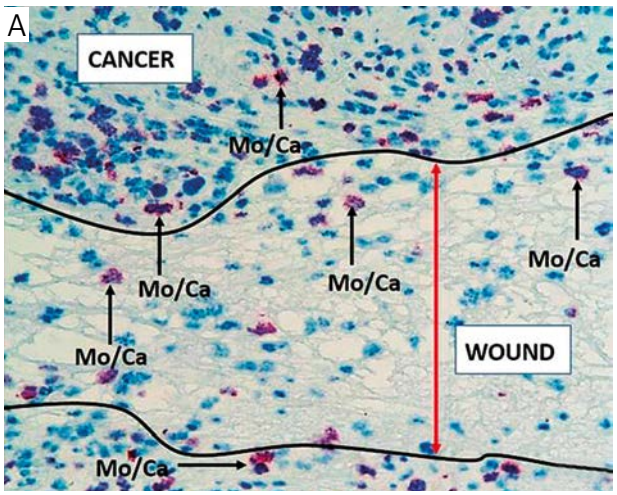

Fig. 4A. Cancer wound. Healing day 7. Staining for BrdU incorporation. Magnification 200×

Mo/Ca – proliferating mononuclear/cancer cells (red stained). Single proliferating cells located mainly on the edge of the wound

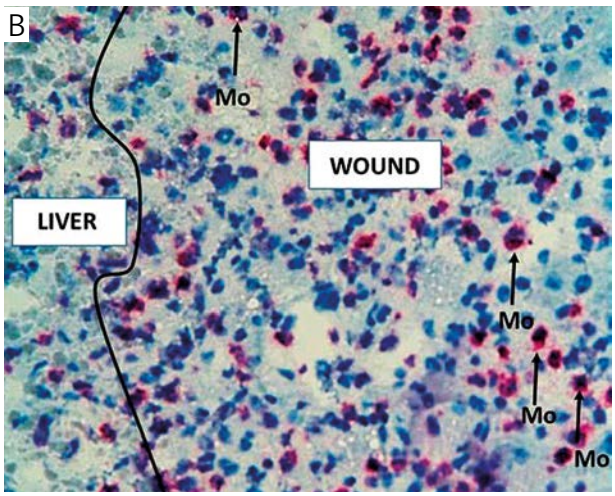

Fig. 4B. Normal liver wound. Healing day 7. Staining for BrdU incorporation. Magnification 200×

Mo – proliferating mononuclear cells infiltrating the wound. Abundant red stained mononuclear cell infiltration located in the wound

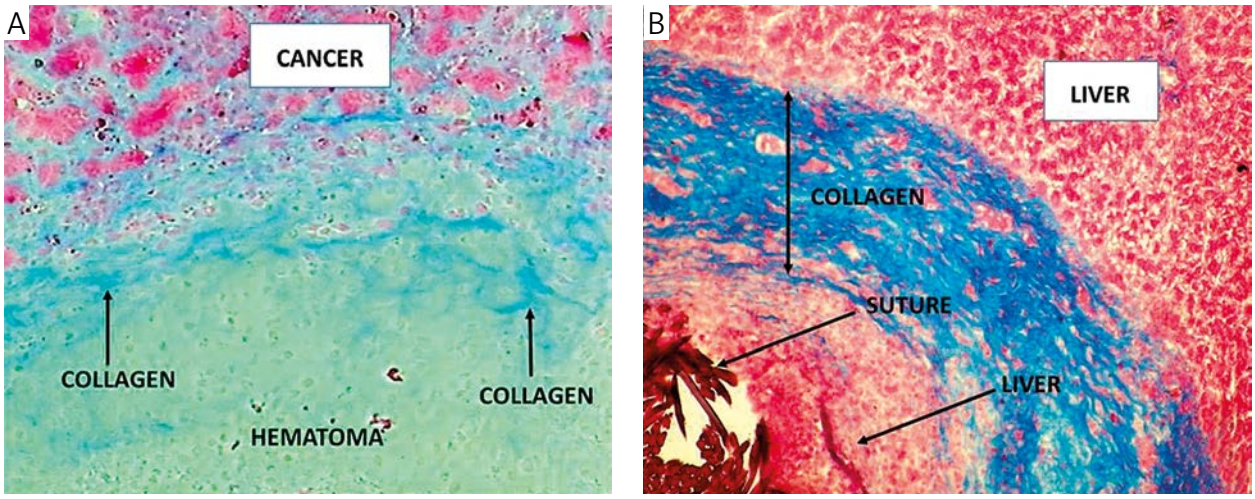

Fig. 5. A) Cancer wound. Healing day 7. Staining for collagen. Magnification 400×. Blue stained collagen fibers are irregular and sparse. **B)** Normal liver wound. Healing day 7. Staining for collagen. Magnification 400×. Blue stained collagen fibers

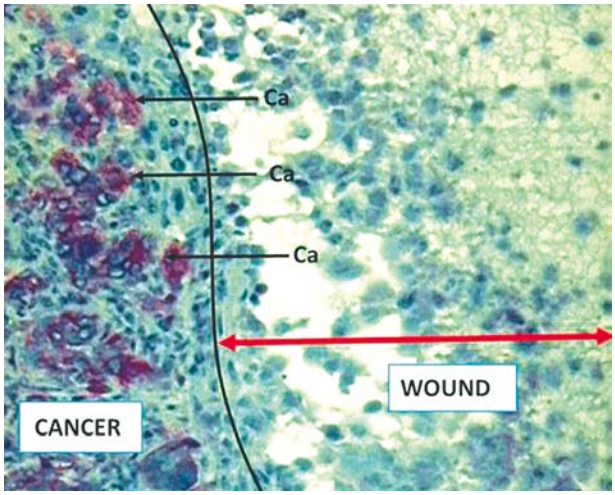

Fig. 6. Cancer wound. Healing day 7. Staining for CC531 cancer cells. Magnification 400×. Red stained cancer cells were infrequently found in the wound site

Table III. Cumulative data of mononuclear infiltration, vessel and connective tissue formation, BrdU incorporation and cancer seeding in the normal vs. cancer wound in the liver

	Cancer wound in the liver	Normal liver wound
Mononuclear infiltrates	+	+++
Vessel formation	+ (single)	+++
Collagen deposition	+	+++
BrdU incorporation	+ (MNC) + (CC531)	+++ (MNC) –
CC531 seeding	+ (single)	–

Cumulative data of mononuclear infiltrations count, vessel and connective tissue formation, BrdU incorporation and cancer seeding in the studied wound models are described in Table III.

Discussion

Our study provided the following findings: 1) the healing of cancer wounds was impaired; 2) the number of wound-infiltrating mononuclear cells (mainly macrophages) was significantly lower in cancer than in normal liver wounds; 3) there were no differences in phenotypes of cells infiltrating cancer and normal liver wounds; 4) BrdU incorporation confirmed the high proliferative potential of cancer but not infiltrating mononuclear cells or fibroblasts; 5) there was significantly lower connective tissue formation with low fibroblast proliferation and poor collagen deposition within the cancer wounds compared to the normal liver wounds; 6) liver cancer wounds were significantly deprived of newly formed blood vessels; 7) there was no migration of CC531 cells into the cancer wounds. Surgical wounding may provide a favorable condition for tumor recurrence at the site of resection or in the abdominal wall [21, 22]. Attention to this issue was revived by a number of authors with regard to local recurrences following anterior resections of colon and rectal cancers. In the majority of instances the recurrences occurred in the anastomotic wound [23, 24]. The mechanical dissemination of cancer cells during surgery has been discussed in the medical literature since the end of the 19th century, and was extensively studied in the 1960s in animal cancer models [25]. Currently, wound 'contamination' by cancer cells at the time of the operation is one of the recognized causes of local recurrence [26, 27]. It has been suggested that deposition of cancer cells, which may be desquamating from the tumor surface, persistent in the peritoneal fluid, or present in circulating blood and transected lymphatics, may

also contribute to some recurrences [1, 28]. Robinson and Hoppe showed that V2 rabbit carcinoma injected into the aorta implanted more frequently in limbs subjected to ischemia or blunt trauma than in normal limbs [14]. There may be two mechanisms behind this observation: 1) cancer cells passing through the traumatized tissue in the blood stream may become mechanically trapped in the wound, and 2) the high cytokine concentration associated with wound healing may attract cancer cells to migrate to the wound and may stimulate cancer seeding and growth. Interestingly, in our study we did not observe migration of CC531 cells from the border of the incised tumor into the cancer wound. Cancer cells in the cancer wounds were single, suggesting that they were mechanically torn away from the tumor mass during incision rather than migrating from the tumor edge. This observation has two explanations: 1) only a small percentage of cancer cells within the tumor nodule are capable of forming metastases; 2) poor macrophage and lymphocyte wound infiltration followed by weak fibroblast migration leads to low concentrations of cytokines, growth factors and extracellular matrix components (ECM) in the cancer wound [29].

We observed impaired healing of the liver cancer wounds with reduced proliferation activity of fibroblasts and poor deposition of collagen, with no connective tissue formation. The action of fibroblasts in the cancer wound may be two-edged. Fibroblasts play a crucial role in scar tissue formation, during the proliferation, repair and remodeling phases of scar formation, but also support the process of stroma formation during tumor growth. Fibroblasts produce a number of growth factors (including FGF, EGF, PDGF and TGF-β), and ECM components (such as collagen, elastin and proteoglycans), which serve in wound and tumor stroma formation. Fibroblasts also produce matrix metalloproteinases and tissue inhibitors of metalloproteinases, which play a crucial role in remodeling and repair of scar tissue and tumor stroma. It has been shown that interactions between tumor cells and normal fibroblasts enhance the invasive and metastatic potential of tumor cells [4, 22].

In our study we observed strong BrdU incorporation by the tumor cells surrounding the cancer wound. This observation suggests high proliferative and metastatic potential of CC531 cells. In this issue, lack of migration of cancer cells into the cancer wound may be explained by their weak stimulation by fibroblasts, macrophages and lymphocytes, rather than the weak metastatic potential of CC531 cells. Poor migration and proliferation of fibroblasts, which result in impaired or even no healing process within the cancer wound, are probably a result of poor infiltration of the wound site by the macrophages and lymphocytes. Although the CC531 adenocarcinoma metastases in the liver are surrounded and heavily infiltrated by lymphocytes and macrophages, only a few of these cells were found to migrate into the wound site. Our study showed a significant difference in the mean count per microscopic field of mononuclear cells infiltrating normal and cancer liver wounds. This observation needs a short explanation of tumor-host interactions within the tumor microenvironment. Inflammatory cells such as neutrophils, eosinophils, lymphocytes and macrophages are affected by a range of physiological and chemotactic factors produced by the tumor. In the tumor microenvironment cancer cells effectively reprogram infiltrating immune cells, to the point where they fail to fight, or even start to support malignant progression. Macrophages possess a multitudinous inventory of functions, and are often described as the 'Swiss army knife' of the immune system. They are recruited through the local expression of chemoattractants such as macrophage colony stimulating factor 1, monocyte chemotactic protein 1, granulocyte/macrophage colony stimulating factor, macrophage inflammatory protein-1-α and macrophage migration inhibitory factor [30]. Macrophages isolated from different anatomical sites showed functional and phenotypic differences. Such differences probably result from the influence of the microenvironment as well as the appropriate activation and nature of the differentiation stimulus [31]. Tumor-associated macrophages (TAMs) are capable of influencing a number of processes, such as matrix remodeling, angiogenesis and stimulation of tumor growth and motility through synthesis of growth and chemotactic factors [32]. TAMs have the potential to carry out both anti-tumor and pro-tumor activities. There is a hypothesis that tumors subvert the normal functions of TAMs in order to promote tumor growth and metastases [33]. Our previous studies on the adherence of mononuclear cells infiltrating CC531 liver tumors revealed a predilection of CD14, MHCII-positive cells (that is TAMs) for liver adenocarcinoma metastases, with the highest propensity being for adherence to tumor stoma [34]. TAMs and wound macrophages have functional similarities to one another, for instance, less cytotoxic activity than activated macrophages, and have the capacity to affect angiogenesis, stroma formation and dissolution [35]. Although there are some functional similarities between TAMs and wound macrophages, it seems that CC531 TAMs which are strongly bound to the tumor tissue lose their migration potential and capacity to participate in the cancer wound healing process. Additionally, stromal fibroblastic hyperplasia around the CC531 tumor may provide an effective barrier against the migration of newly recruited peripheral macrophages stimulated by the tumor wounding process. Angiogenesis is marked by endothelial cell migration and capillary formation in the proliferative healing phase.

Capillaries supply nutrients for granulation and tissue deposition, and failure of this results in lack of healing. In our study, cancer wounds in the liver were deprived of newly formed capillaries, whereas in normal liver incisional wounds blood capillaries were abundant.

Neovascularization plays a crucial role in successful wound healing, and is probably regulated by FGF-2 and VEGF [36]. A number of leukocytes secreting bFGF, tumor necrosis factor, and VEGF were shown to be associated with tumor angiogenesis [37]. Tumor-infiltrating leukocytes are often induced by the cancer microenvironment to display a pro-tumor, pro-angiogenic phenotype. This "polarization" has been described for several myeloid cells, in particular macrophages, which contribute to the process of angiogenesis by secreting pro-angiogenic mediators, including basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator, and adrenomedullin, to facilitate tumor angiogenesis [38, 39]. Another population of innate immune cells able to infiltrate tumors is that of natural killer (NK) cells. The role of NK cells in tumor progression and angiogenesis has not yet been fully investigated, but recent data have suggested that they are potentially pro-tumorigenic and can also acquire a pro-angiogenic phenotype [40]. Poor mononuclear infiltration of cancer wounds explains the lack of newly formed capillaries.

Conclusions

Presence of cancer at the surgical margin is rarely encountered today, as the principles of negative proximal and distal margins are well appreciated.

During palliative R1 and R2 excisions, cancer cells may persist or surround the healing wound. Moreover, in our previous study we documented that wounds may become sites of intensive proliferation of tumor cells and cause wound dehiscence [21].

This study confirmed that the impaired migration and proliferation of inflammatory cells in cancer wounds followed by poor scar tissue formation contributes to impaired healing of cancer 'contaminated' wounds.

The authors declare no conflict of interest.

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