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Tumour seeding in peritoneal wound sites in relation to growth-factor expression in early granulation tissue

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Abstract

The purpose of these experiments was to identify growth factors produced during the formation of a peritoneal wound in relation to tumour cell seeding and stimulated growth in granulation tissue. Gelfoam© gelatin sponge was implanted in the mesenteric fan of nude mice to initiate the granulation process. Human HT29 colon carcinoma cells were inoculated intraperitoneally at various times after sponge implantation and tumour growth in granulation tissue was determined. RNA isolated from granulation tissue was used for polymerase chain reaction analysis of the expression of specific growth factors and receptors [vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and lysophosphatic acid (LPA)], and for microarray analysis of differentially expressed genes in early vs. late granulation tissue. Inflammatory cells infiltrated the sponge within 1 day, followed by fibroblasts and the formation of an extracellular matrix. Tumour cell inoculation at 8 h to 3 days after sponge implantation resulted in extensive tumour formation in all cases. Inoculation at 10–28 days resulted in focal tumour growth in only 16–33% of the sponges. Low amounts of VEGF, TGF-β_{1-3}, TGF-β RIII and LPA receptors 1,2 were detected in early granulation tissue, with increased expression from day 10. Microarray analysis identified additional differentially expressed genes that may stimulate tumour take and growth in early granulation tissue.

Keywords: Tumour seeding; Granulation tissue; Growth factors

1. Introduction

The undisturbed peritoneum constitutes a non-adhesive, non-thrombotic surface, which presents a selective barrier regulating transport between the circulation and the body cavity. During surgery for abdominal cancer this barrier is disturbed and tumour cell seeding at catheter port sites and in surgical scar tissue is well documented [1,2]. The short time interval before these recurrences are detected (median 109 days for colorectal cancer) suggests massive cell seeding in the abdominal wall during surgery [3]. Experimental models have also clearly demonstrated a high incidence of tumour seeding at peritoneal wound sites [4–6]. Once peritoneal spread has occurred, survival is poor. For colorectal cancer the median survival is only 10–12 months, despite systemic chemotherapy. A greater understanding of the processes leading to tumour cell seeding in peritoneal wound sites should help in designing strategies to inhibit this.

The composition of tumour stroma is strikingly similar to that found in a healing wound and several processes involved in wound healing also play a part, although aberrantly regulated, in tumour progression [7]. For example, transforming growth factor-β (TGF-β) is one of the major factors regulating both processes and its involvement in peritoneal wound healing has been documented [8]. Vascular endothelial growth factor (VEGF) plays a critical part in angiogenesis and is highly expressed both during wound healing and in many tumour types [9]. High expression of VEGF is associated with poor prognosis and increased metastasis

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in both colorectal and ovarian tumours. Lysophosphatic acid (LPA) is a platelet-derived mediator that stimulates cell migration, invasion and proliferation, through activation of its cognate G protein-coupled receptors LPA_1, LPA_2 and LPA_3. LPA promotes wound healing both in vitro and in vivo [10,11] and its receptors are overexpressed in various tumour cells, including those derived from colorectal and ovarian cancers [12]. Increased tumour seeding and growth in wounds is likely to depend on interactions between the tumour cells and the extracellular matrix (ECM) in the wound. These interactions are influenced by metalloproteases, and other proteases that remodel the matrix and detach growth factors from the matrix, as well as by plasmin.

In order to study these processes in detail we set up a model for peritoneal granulation tissue in mice. Granulation tissue can be initiated by implantation of gelatin sponges, which induce a host reaction that resembles a wound healing process. Such models have previously been used to study in vivo mechanisms of angiogenesis [13], characterisation of the wound healing process [14] and host–tumour interactions [15,16]. However, tumour invasion in relation to growth factors present in a healing wound has not previously been studied. The purpose of our work was to establish a peritoneal granulation model for studying the implantation and growth of tumour cells in relation to the expression of various growth factors and receptors produced by the healing wound.

2. Material and methods

2.1. Mice

Female nude BALB/c (nu/nu) mice were obtained from the Animal Department of the Netherlands Cancer Institute. Sterilised food and water was provided ad libitum. The animals were 68 weeks old and weighed 2024 g at the time of experiment. All animal studies were approved by the local animal experimentation committee and were carried out in compliance with national guidelines for the care and use of research animals.

2.2. Tumour cell lines

Human colorectal carcinoma cells (HT29) were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum (FCS) (Gibco, Breda, The Netherlands), penicillin (100 U/ml) and streptomycin (100 U/ml).

2.3. Peritoneal injury

Mice were anaesthetised by intraperitoneal (i.p.) injection of a mixture of Hypnorm (fentanyl 0.04 mg/kg; fluanosine 1.25 mg/kg) and Dormicum (midazolane 0.625 mg/kg). After disinfecting the abdominal skin, a small incision (0.5 cm) was made in the skin and abdominal wall. Subsequently only the skin was sutured, leaving an open peritoneal wound. After 1 h the mice ($n = 6$ per group) were inoculated with increasing numbers of HT29 (10^5–10^6) tumour cells (injected i.p. in 200 μl phosphate-buffered saline). With this model we aimed to mimic the clinical condition in which tumour cell spillage in the abdominal cavity may occur during surgery. Control mice ($n = 6$ per group), without a peritoneal wound, were also inoculated with the same numbers of tumour cells. After 4 weeks all animals were killed and the macroscopic abdominal tumour take was determined. The TD_{50} (take dose; estimated number of cells required to produce macroscopically visible tumour in 50% mice) was calculated from these data by probit analysis. The tumour load was also recorded (in arbitrary units, a.u.) on standardised forms indicating very large (11–15 mm: 4 points), large (6–10 mm: 3 points), moderate (1–5 mm: 2 points), small (<1 mm: 1 point) or no tumour (0 points) in seven abdominal regions (pelvis, omentum, transverse colon, mesentery, left and right subdiaphragmatic area, subhepatic area) and at the wound site.

2.4. Gelatin sponge implantation

Sterile Gelfoam® gelatin sponges (Pharmacia & Upjohn, Kalamazoo, USA) were cut into small pieces ($5 \times 5 \times 4$ mm) and immersed in sterile saline. Mice were anaesthetised by injection (i.p.) of a mixture of Hypnorm and Dormicum, 10 min before implantation. After disinfecting the abdominal skin, a small incision (0.5 cm) was made in the abdominal wall. A piece of gelatin sponge was then implanted in a small opening made in the mesenteric fan of the animals. The abdominal wall and skin were sutured separately. After 8 h, 1, 2, 3, 7, 10, 14, 21 or 28 days mice ($n = 6$ per group) were killed and sponge tissue was removed and fixed in 4% formaldehyde or snap frozen in liquid nitrogen. Paraffin sections of the sponge tissue were stained with haematoxylin/eosin (H&E) for histological analysis. Snap-frozen sponge tissues were used for RNA isolation and subsequent polymerase chain reaction (PCR) or microarray analysis.

2.5. Tumour growth in granulation tissue

At 8 h, 1, 2, 3, 7, 10, 14, 21, or 28 days after sponge implantation mice ($n = 6$ per group) were inoculated with 1×10^6 HT29 tumour cells (i.p.). The mice were killed 28 days after the inoculation and intra-abdominal tumour load was determined. Sponge tissues were removed and fixed in 4% formaldehyde. Paraffin sections

were stained with H&E and two sections of each sponge tissue were examined for tumour formation.

A control experiment was performed to examine whether tumour cell invasion is physically inhibited by the presence of an extracellular collagen and fibrinogen matrix in a more mature granulation tissue. For these experiments, gelatin sponges were harvested 21 days after implantation and transplanted to a new group of mice to reinduce the wound healing process. After 1 or 10 days these mice were inoculated with HT29 tumour cells (i.p.) and tumour growth in the granulation tissue was determined as described above.

2.6. RNA isolation and PCR analysis

Frozen sponge tissues were homogenised in TRIzol® reagent (Life Technologies) using a Polytron PT1200 power homogeniser (Kinematica). Total RNA was then isolated using TRIzol® reagent, according to the manufacturer's protocol. RNA was reverse-transcribed to first-strand cDNA with random hexamer primers (Applied Biosystems) and Superscript II reverse transcriptase (Invitrogen Life Technologies). Expression of VEGF and LPA receptors 1, 2 and 3 was determined. The primers used for VEGF were 5′-CTGTAACGATG AAGCCCTGG-3′ (forward) and 5′-TGCTGTAGGAA GCTCATTCTCTC-3′ (reverse) (Sigma Genosys Ltd.). For LPA_1, the forward primer 5′-GTCAACCGCCGC TTCCATTT-3′ and the reverse primer 5′-GTTGAA AATGGCCCAGAAGA-3′ were used. For LPA_2, 5′-CCCCGCTACCGAGAGACCAC-3′ (forward) and 5′-AAAGGGTGGAGTCCATCAGT-3′ (reverse), and for LPA_3, 5′-AATTGCCTCTGCAACATCTC-3′ (forward) and 5′-TACATGTCCTCGTCCTTGTA-3′ (reverse), were used. GAPDH was used for normalisation to correct for the variation in product yield. The primers used for GAPDH were 5′-TGCACCACCAACTGCTT AG-3′ (forward) and 5′-GGATGCAGGGATGATG TTC-3′ (reverse). The optimised PCR conditions for VEGF and the LPA receptors were as follows: 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min followed by an extension at 72 °C for 7 min.

Expression of $TGF\beta_{1,-2,-3}$ and TGF-β receptorsI, -II and GAPDH was determined using a multiplex PCR kit from Maxim Biotech, Inc, USA, according to the manufacturer's protocol. The optimised PCR condition was two cycles of 96 °C for 1 min and 57 °C for 4 min followed by 30 cycles of 94 °C for 1 min and 57 °C for 2.5 min, and an extension at 70 °C for 10 min. Each PCR experiment was performed three times to get weighted averages of expression.

The PCR products were separated on a 2% agarose gel. The presence and the intensity of each band were compared, using Tina 2.0, for sponge tissues harvested at various times after implantation.

2.7. Microarray experiments

Total RNA from granulation tissue of 3, 10 and 28 days post-implantation was linearly amplified using the following protocol. RNA was reverse transcribed using a synthetic primer containing the poly-A and T7 RNA polymerase binding site, 5′-GGCCAGTGAATTGTA ATACGACTCACTATAGGGAGGCGG$(T)_{24}$-3′ (Sigma Genosys). Second-strand cDNA synthesis was performed with RNAse H, *Escherichia coli* DNA polymerase I and *E. coli* DNA ligase (New Engl. Bio. Labs). The double-stranded cDNA was then purified with a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol and transcribed with T7 polymerase (T7 Megascript kit; Ambion, Austin, TX) for 14–16 h at 37 °C to yield linearly amplified antisense RNA. The amplified RNA (aRNA) was purified with RNeasy mini-columns (Qiagen) and used for a second round of amplification.

aRNA harvested from sponges at 28 days post-implantation was used as the reference for cDNA microarray analysis. The mouse cDNA microarray chips contained 15000 spotted cDNAs and were manufactured at the Netherlands Cancer Institute microarray facility. aRNA (2 μg) was reverse transcribed and directly labelled with cyanine 5-conjugated (Cy-5) deoxyuridine 5′-triphosphate (dUTP) (reference sample: day-28 granulation tissue) or cyanine 3-conjugated (Cy-3) dUTP (test samples: day 3 or 10 granulation tissue) (CyeDye; Amersham Pharmacia Biotech). Cy-3- and Cy-5-labelled cDNA was combined and purified with the Qiaquick PCR purification kit (Qiagen). Microarray slides were prehybridised in $5\times$ standard saline citrate (SSC), 1% bovine serum albumin and 0.1% sodium dodecyl sulphate (SDS) for 1–2 h at 42 °C.

Hybridisation was performed in $10\times$ SSC, 25% formamide and 0.02 % SDS for 14–16 h at 42 °C. After hybridisation, slides were scanned with a SA4000L scanner (Gsi Limonics). For each experiment, four sets of hybridisations were used. For the straight-colour hybridisation, the test (days 3 and 10) and control aRNA (day 28) were labelled with Cy-3 or Cy-5, respectively. A reverse-colour hybridisation was also performed with test aRNA labelled with Cy-5 and control aRNA labelled with Cy-3. In addition a self–self hybridisation for each of the two samples was performed for each experiment. For this, aRNA of each sample was labelled with either Cy-3 or Cy-5, after which a microarray was hybridised with labelled cDNA of the same sample. Each experiment, consisting of four hybridisations, was performed twice. A detailed protocol for RNA amplification, cDNA probe labelling and hybridisation is available at http://microarrays.nki.nl/home.html [The Netherlands Cancer Institute (NKI) microarray facility].

2.8. Microarray data analysis

Imagene™ 4.2 software (BioDiscovery, Inc.) was used to calculate the spot intensities of the Cy-3 and Cy-5 images obtained after scanning. These raw data were then uploaded in the web-based MicroArray Bioinformatics program of the NKI microarray facility (program developed by A. Velds). The images of each array were normalised, to correct for unequal incorporation of the Cy-3 or Cy-5 labels or an unequal amount of the aRNA samples, and straight-colour and colour-reverse images were combined to get a weighted average. Genes that appeared as differentially expressed in the self–self experiments were eliminated from the final analysis. For this study, $P < 0.01$ was used to define differential gene expression to minimise false-positive elements.

3. Results

3.1. Effect of peritoneal injury on tumour growth

Peritoneal injury 1 h before tumour cell inoculation led to fewer tumour cells being required to induce intra-abdominal tumour formation. Macroscopic peritoneal tumours were observed in some animals at 4 weeks after inoculation of 10^5 HT29 cells, whereas in control mice tumours were only seen after inoculation of at least 4×10^5 cells (Fig. 1). The estimated TD_{50} (tumour cell number required to induce tumours in 50% mice) was significantly lower in animals with a peritoneal wound than in control animals (2.8×10^5 vs. 5.4×10^5, $P < 0.025$) (Fig. 1). Tumours were always found at the peritoneal wound site in tumour-bearing mice and these tumours were always larger (2–5 times) than tumours formed at the other intra-abdominal sites. This indicates that peritoneal wounding induced not only an increase in tumour take, but also in tumour growth. Similar results were obtained using a human ovarian carcinoma cell line (IGROV) (TD_{50} of 4.7×10^5 and 12×10^5 in wounded and control mice, respectively; data not shown).

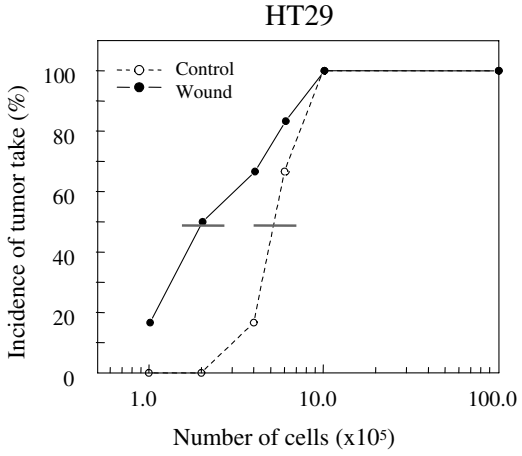

Fig. 1. Percentages of intra-abdominal tumour take in control and wounded mice. Animals were inoculated intraperitoneally with increasing numbers of HT29 cells and tumour take was determined after 4 weeks. Data represent percentage of tumour take (any visible tumour in six mice per group). The horizontal bar indicates the TD_{50}.

3.2. Granulation tissue induction in implanted gelatin sponge

Pieces of sterile gelatin sponges were implanted in mice to induce formation of granulation tissue. Some inflammatory cells, mainly macrophages and eosinophilic granulocytes, had already infiltrated the sponge matrix at 8 h after implantation. After 1–3 days more inflammatory cells were present, including lymphocytes as well as granulocytes and macrophages (Fig. 2(d)). Erythrocytes were also seen in the sponge matrix in the first 4 days after implantation. The initial inflammatory response was followed by the infiltration of fibroblasts and the formation of an ECM (Fig. 2(e)). At 21–28 days post-implantation, a complete granulation tissue was formed in the sponge matrix, which consisted of mainly fibroblasts, some inflammatory cells and well-formed blood vessels, lined with endothelial cells and filled with red blood cells, in an ECM (Fig. 2(f)).

3.3. Tumour growth in granulation tissue

Abdominal tumour load and tumour growth within the implanted sponge matrices was examined at 28 days after tumour cell inoculation. There was no significant difference between the different groups of mice in abdominal tumour growth outside the sponge tumour matrix (Table 1). However, large differences were seen in tumour load within the sponge matrix, depending on the maturity of the granulation tissue at the time of tumour cell inoculation. Inoculation of HT29 cells from 8 h to 3 days after sponge implantation induced tumour formation in the granulation tissue in 83–100% of the animals (Table 1). In these animals the sponge matrix was completely replaced by viable tumour cells (Fig. 3(a)). Tumour cell inoculation at 7 days after sponge implantation also induced tumour take in 83% of the cases (Table 1), although in these animals only small tumour areas were formed in the sponge matrix (Fig. 3(b)). As the interval between sponge implantation and tumour cell inoculation was increased to 10 days or more, the tumour take in the granulation tissue decreased to only 16–33% of mice (Table 1), in which only small tumour areas were formed within the sponge tissue. The majority of the implanted sponge tissues in this group was free of tumour cells but contained normal granulation tissue (Fig. 3(c)), as was seen in animals that were not inoculated with tumour cells.

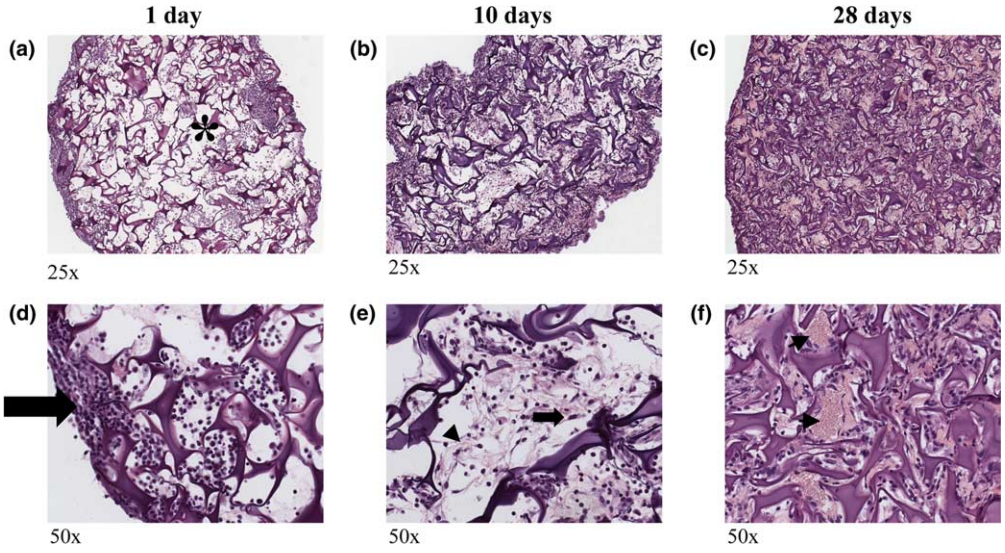

Fig. 2. The formation of a granulation tissue in time in implanted gelatin sponges. (d), (e) and (f) (magnification 50×) are a higher magnification of (a), (b) and (c), respectively, (magnification 25×) (all H&E-stained sections). Inflammatory cells (arrow) had already infiltrated into the sponge matrix (*) (d) at 1 day after implantation. (b) and (e) show infiltrated fibroblasts (arrow) and extracellular matrix (arrowhead) formed at day 10. At 21 days after implantation, a complete granulation tissue had formed, including blood vessels (arrows) (c, e).

Table 1
Abdominal tumour growth and tumour take in granulation tissue in relation to the time interval between sponge implantation and cell inoculation

Time interval (days)	Abdominal tumour load (a.u.)	Tumour take (%) in sponge
0.3	11.5 ± 4.1	100[a]
1	6.3 ± 5.0	100[a]
2	9.5 ± 3.1	83[a]
3	8.5 ± 2.1	100[a]
7	9.7 ± 2.4	83[b]
10	6.0 ± 3.1	16.7[b]
28	6.8 ± 2.4	33.3[b]

The mean abdominal tumour load (±SD) as determined by semi-quantitative tumour scoring is represented in arbitrary units (a.u.) indicating the amount and size of the abdominal tumours ($n = 6$ animals per group, two sections per animal) at 4 weeks after inoculation of tumour cells.

Tumour take in sponge represents the % of animals that had any visible tumour in the implanted granulation tissue ($n = 6$ animals per group).

[a] Sponge matrices completely filled with tumour.

[b] <25% of sponge matrix filled with tumour.

Tumour growth in a reimplanted 21-day granulation tissue was also determined. This once again revealed that tumour cell inoculation at 1 day after reimplantation of the preformed granulation matrix resulted in tumour formation in 75% of the sponges, with an almost complete replacement of the granulation tissue by vital tumour tissue (>80% of the sponge). Inoculation of HT29 tumour cells at day 10 after the implantation of a 21-day granulation tissue did not result in tumour formation in the granulation tissue. This further demonstrates that the host reaction induced by sponge implantation, rather than the composition of the sponge tissue at time of implantation, is important for the induction of tumour formation.

3.4. Expression of VEGF, LPA receptors and TGFβ in granulation tissue

Total RNA yields were approximately 10 μg from a granulation tissue of up to 10 days, and 20 μg from

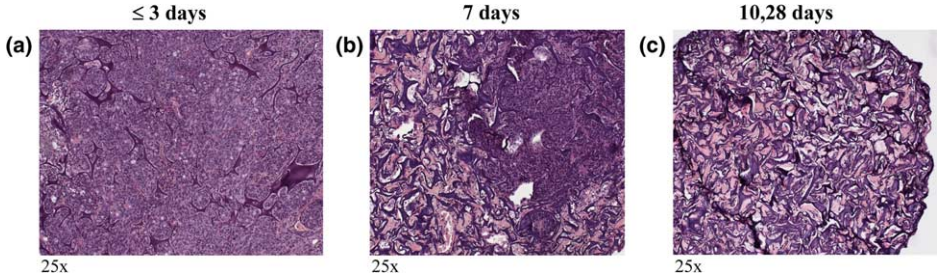

Fig. 3. Tumour formation in granulation tissue formed in implanted gelatin sponges. Inoculation of 10^6 HT29 cells during the first 3 days after sponge implantation resulted in complete replacement of sponge matrix by viable tumour tissue (a). Only small tumour areas (<25%) were formed in the sponge matrix when tumour cells were inoculated 7 days after sponge implantation (b). Inoculation at 10 days or later, the majority of the sponges contained only granulation tissue, without tumour (c). H&E; 25×.

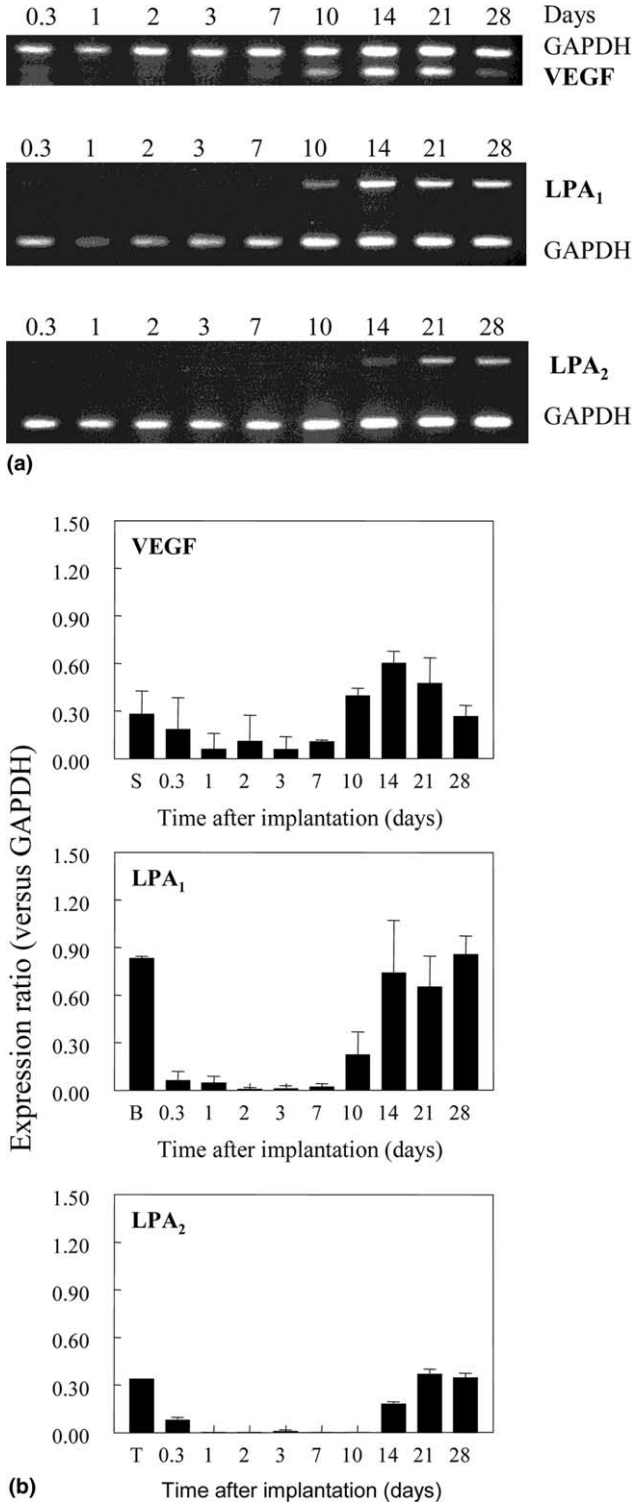

Fig. 4. Polymerase chain reaction analysis of growth-factor expression in maturing granulation tissue. RNA was isolated from granulation tissue of 8 h (0.3 days), 1, 3, 7, 10, 14, 21 and 28 days after sponge implantation. RNA expression of vascular endothelial growth factor (VEGF), and lysophosphatic acid (LPA) receptors 1 and 2 (a, b) was determined. Spleen (S), brain (B) and testis (T) were used as positive controls. In (a) the expression ratios for each factor corrected for the expression of the housekeeping gene GAPDH is shown. The mean ± SD of three experiments is shown.

granulation tissue from 14 to 28 days post-implantation. Results are shown as expression ratio of genes of interest relative to GAPDH. RNA from a positive control tissue was included for each PCR, i.e., spleen (VEGF), brain (LPA_1 and TGFβ) and testis (LPA_2).

VEGF expression was low in granulation tissue of up to 7 days, with increased expression from day 1021 and subsequent decrease by 28 days (Fig. 4(a) and (b)). Expression of the LPA receptors LPA_1 and LPA_2 was also low during the first 7 and 10 days, respectively, after which it increased (Fig. 4(a) and (b)). An overall higher expression of LPA_1 receptor than LPA_2 receptor was seen in late granulation tissue. LPA_3 receptor was not expressed in the granulation tissue. Expression of TGF-$\beta_{1,-2,-3}$ and TGF-β receptors I and II was low in the granulation tissue from 1 to 7 days after sponge implantation. At later times (from 10 days) there was an increased expression of all factors and receptors (Fig. 5). Expression at 8 h was mostly very low, although expression of both TGF-β_1 and TGFβ RII was seen at this time.

3.5. Microarray analysis of gene expression

Gene expression in maturing granulation tissue was also determined by microarray analysis. A comparison of the straight-colour and colour-reverse hybridisations of early (3 days) and mature (day 28) granulation tissue (Fig. 6) revealed differential expression of 274 genes in the early granulation tissue. Of the 244 genes with an increased expression, 14 were associated with inflammatory and/or immune responses, five with blood coagulation, two with adhesion and three with ECM formation (Table 2 shows a selection of these genes; a complete list is available on request). The remaining upregulated genes were not known to be involved in wound healing responses, or were expressed sequence tags (list available on request).

Of the 30 genes with a decreased expression, three were involved in inflammation or the immune response, two in cell migration and two in ECM formation [e.g., inhibitor of metalloproteinase (TIMP-2)] (Table 2). The self–self hybridisations of these experiments revealed differential expression of only two genes (Fig. 6(b)), which were eliminated from further analysis.

Subsequently, the differentially expressed genes in early and intermediate granulation tissue were compared to study the expression pattern of the genes during granulation tissue maturation. The expression of a total of 76 genes was increased in early (3 days), but not in intermediate (day 10) granulation tissue, of which 29 genes have a known function. Of these 29 genes, several are involved in inflammation and immune response, blood coagulation and one gene, *melanoma x-actin*, has a role in cell adhesion (Table 2 shows a selection of these

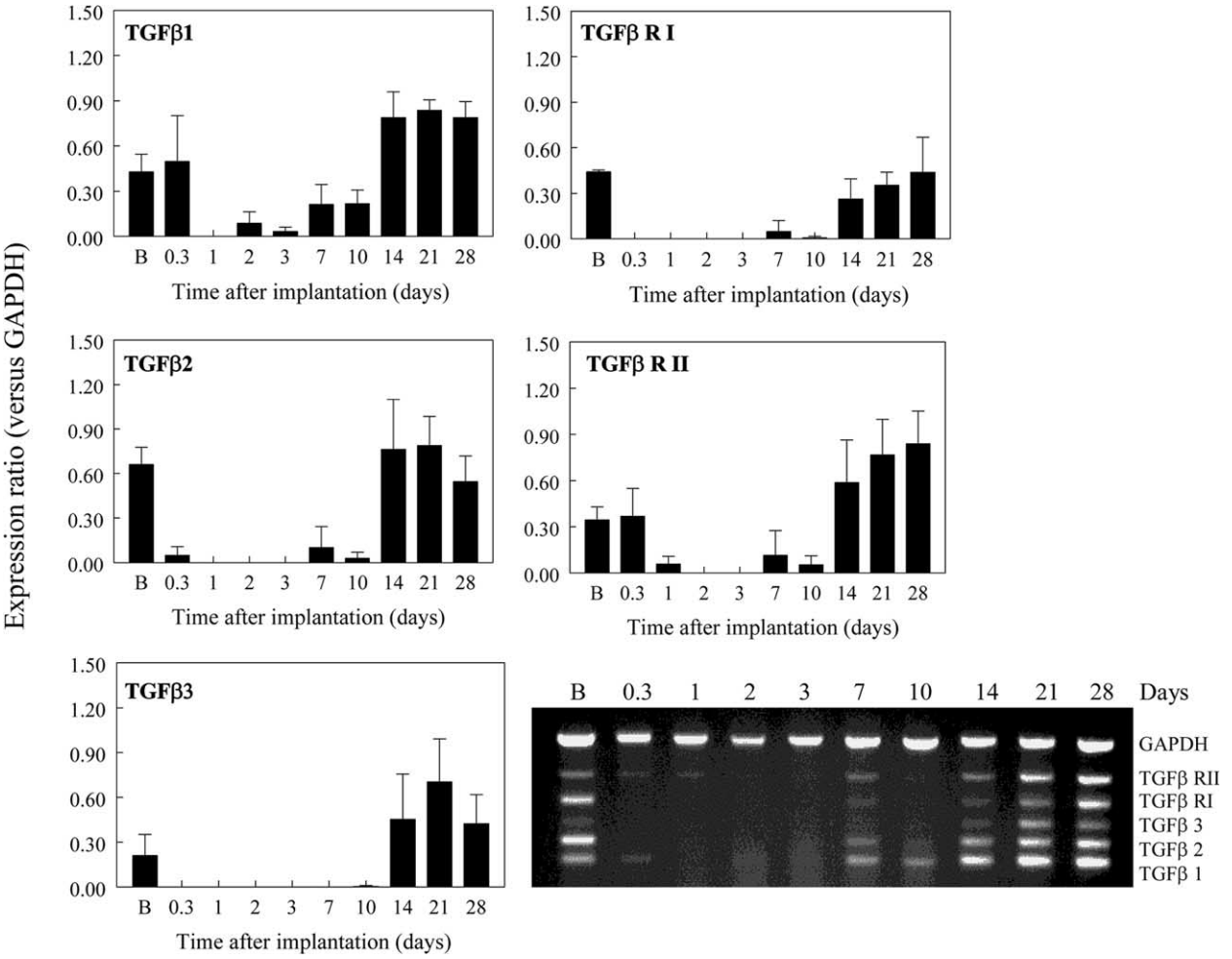

Fig. 5. Multiplex polymerase chain reaction analysis of transforming growth factor-$\beta_{1,2,3}$ (TGFβ) and TGFβ receptors I and II expression in maturing granulation tissue. RNA was isolated from granulation tissue of 8 h (0.3 days), 1, 3, 7, 10, 14, 21 and 28 days after sponge implantation and brain (B) tissue was used as a positive control.

genes). These genes are all candidates for further investigation with respect to stimulation of tumour growth in early granulation tissue.

Several genes had increased expression in both early and intermediate granulation tissues, including *CXCR-4* and *RhoB*, which are both involved in cell migration, and *Jagged-1*, which is involved in angiogenesis and remodelling of damaged arteries. Another gene with increased expression in both early and intermediate granulation tissue was that for bone morphogenetic protein (BMP-1).

4. Discussion

This study demonstrates that the presence of a peritoneal wound promotes tumour cell seeding and growth, which is consistent with earlier reports [6,16–18]. Our experiments also show that stimulated tumour seeding and growth were more marked in early (day 3) granulation tissue than intermediate (day 10) or late (day 28) granulation tissue. Tumour cells were not inherently unable to invade into mature granulation tissue, since infiltration did occur in retransplanted, mature granulation tissue. These results suggest that the inflammatory cells present in early granulation tissue, and their production of various growth factors, may be an important factor in stimulating tumour cell invasion and growth in the wound.

It is known that several growth factors, including VEGF, LPA, TGF-β and metalloproteases, have an important role during both wound healing and tumour formation and progression [8,12]. However, the time course for the expression of these growth factors in maturing granulation tissue has not been previously investigated. The goal of our research was to study these events in relation to tumour formation in peritoneal wounds. Granulation tissue produced by peritoneal implantation of gelatin sponge proved to be an effective method for studying these processes.

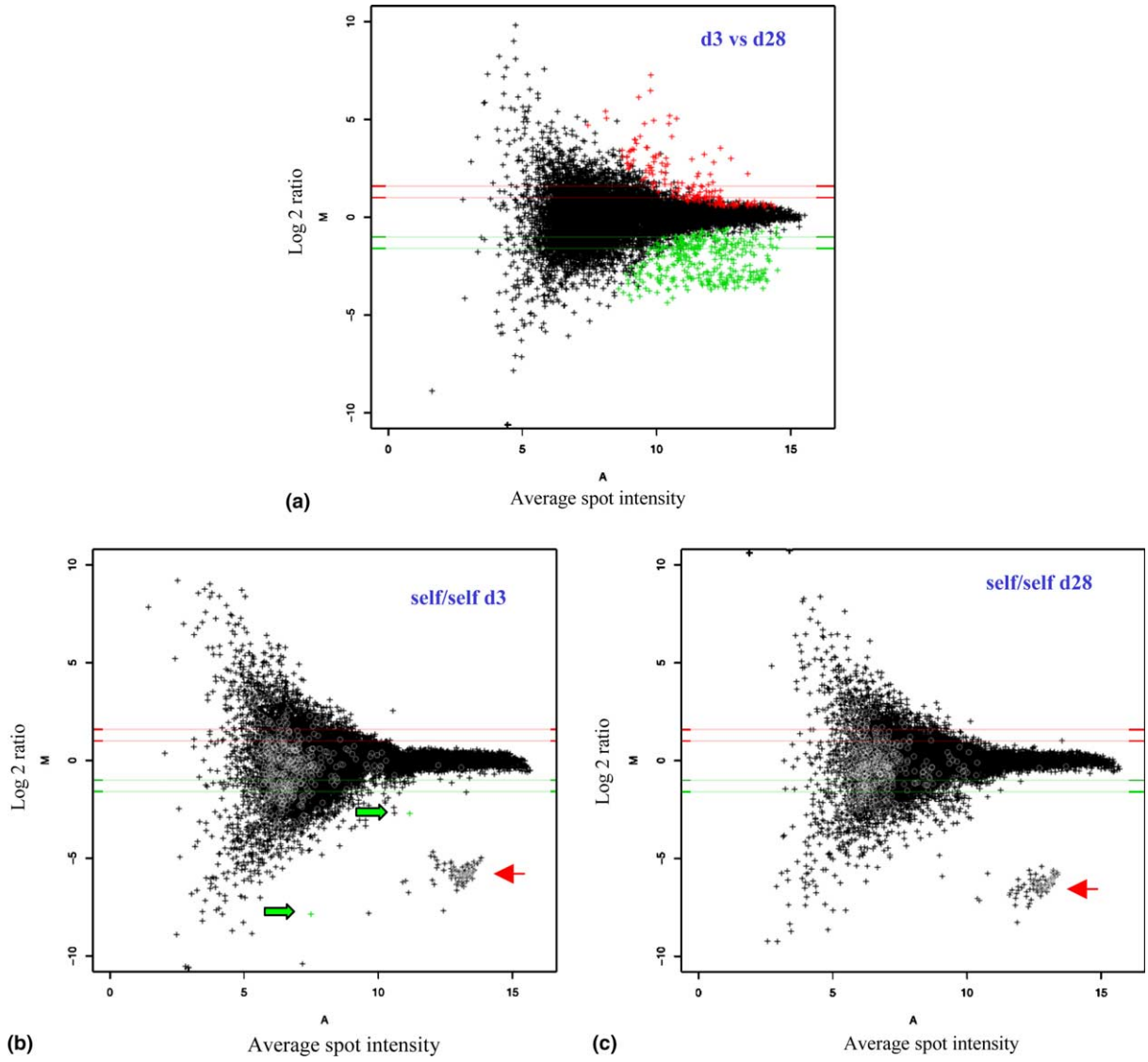

Fig. 6. Error plots of the combined straight-colour and colour-reverse hybridisations and self–self hybridisations of aRNA from early (day 3) vs. late (day 28) granulation tissue. The plots show intensity (*A*) of each cDNA spot vs. the $\log_2$ (M) of the cyanine (Cy)-5/Cy-3 ratios. In the straight-colour hybridisation test, amplified RNA (aRNA) (days 3) was labelled with Cy-3 and control aRNA (day 28) with Cy-5. The green spots indicate genes whose expression is significantly increased and the red spots genes whose expression is significantly decreased in the early granulation tissue. For the self–self hybridisations, aRNA from both early (day 3) and late (28 days) granulation tissue was labelled with Cy-3 or -5, after which the same samples (day 3–day 3 or days 28–days 28) were combined for hybridisation of the microarray. The green spots are genes that are differentially expressed (green arrows). The region of spots with a negative ratio and high spot intensity are marker genes (red arrows), which are only hybridised with Cy-3. These genes are spotted on the slides as positive controls for the hybridisation.

The expression of VEGF, LPA receptors and TGF-$\beta_{-1,-2,-3}$ and TGF-β receptors I and II was analysed in RNA isolated from granulation tissue of varying maturity to determine whether one or more of these factors were highly expressed during periods permissive to tumour seeding and growth. PCR analysis showed, however, that there was low expression of these growth factors and receptors during the first week, with increased amounts from 7 days onwards. It is conceivable that, although their expression was low, these factors might still be important in stimulating tumour cell invasion and implantation in the early granulation tissue. This hypothesis could be investigated by injecting inhibitors of VEGF, TGF-β and LPA together with tumour cells in mice with an early granulation tissue. It is also possible that other, as yet unidentified, growth or adhesion factors are highly expressed in the granulation tissue, and are involved in the tumour formation.

In an attempt to identify other candidate genes involved in tumour cell invasion and growth, we compared the gene-expression profiles of early and late

Table 2
Comparison of differentially expressed genes in early only (day 3) or in both early and intermediate (day 10) granulation tissue

Accession No.	Gene	Function	Expression
BG073301	FK506-binding protein 3	Inflammatory/immune	Upregulated 3 days
BG072156	Interferon-inducible protein 16	Inflammatory/immune	Upregulated 3 days
BG072156	Interferon-inducible protein 9–27	Inflammatory/immune	Upregulated 3 days
BG072793	T-cell-activating protein	Inflammatory/immune	Upregulated 3 days
BG074330	Decay-accelerating factor	Inflammatory/immune	Upregulated 3 days
BG072632	Myadm	Inflammatory/immune	Upregulated 3 days
BG072342	Coagulation factor C	Blood coagulation	Upregulated 3 days
BG064672	Endothelial cell protein C receptor	Blood coagulation	Upregulated 3 days
BG075868	Heme-binding protein	Blood coagulation	Upregulated 3 days
BG065030	GSHPx	Blood coagulation	Upregulated 3 days
BG063855	Melanoma X-actin	Cell adhesion	Upregulated 3 days
BG072675	KappaB-Ras1	Immune response	Upregulated 3/10 days
BG076893	RhoB	Cell adhesion	Upregulated 3/10 days
BG063365	CXCR-4	Cell migration	Upregulated 3/10 days
AW538671	Procollagen type V, $\alpha 3$	ECM formation	Upregulated 3/10 days
BG085595	Bone morphogenetic protein 1	ECM formation	Upregulated 3/10 days
BG064712	Growth factor receptor-bound protein 2	Cell proliferation	Upregulated 3/10 days
BG082322	Jagged-1 (JAG-1)	Angiogenesis	Upregulated 3/10 days
lBG077878	Caldesmon II	Cell adhesion	Downregulated 3 days
BG087142	Procollagen type I	ECM formation	Downregulated 3 days
BG088451	TIMP-2	ECM/cell migration	Downregulated 3 days

ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase.

granulation tissue using microarray techniques. Several genes involved in inflammatory processes and blood coagulation were highly expressed in granulation tissue 3 days but not 10 days after implantation, e.g., T-cell-activating protein, interferon-inducible protein, coagulation factor C and endothelial cell protein C receptor. In addition, TIMP-2 expression was decreased in early, but not intermediate or late, granulation tissue. A well-known function of TIMP-2 is the inhibition of metalloproteinases, thereby decreasing the degradation of ECM [19], but it also inhibits tumour invasion and growth [20]. Further studies with specific inhibitors are needed to establish whether there is a causal role for any of these genes in tumour seeding and growth in granulation tissue. If a causal link between any of these growth factors and tumour seeding were established, this would be valuable information in designing specific intervention strategies, possibly using a cocktail of several inhibitors during the immediate postsurgical period.

Another significant observation of this microarray study was the increased expression of several genes in both early and intermediate granulation tissue. One of these, *CXCR-4*, is a chemokine receptor involved in the chemotaxis of cells during inflammation [21], which is involved in metastasis of CXCR-4$^+$ tumour cells [22]. *RhoB* expression was also increased in maturing granulation tissue. This is an endosomal small GTPase involved in oncogenic cell transformation [23,24].The high expression of these genes probably reflect the presence and/or activation state of inflammatory cells, such as macrophages, that may affect tumour growth either by direct contact or by the production by a multitude of stimulatory factors. BMP-1, which was also upregulated in maturing granulation tissue, is involved in the formation of a functional ECM by procollagen processing [25]. It also stimulates cell recruitment during wound healing [26].

In summary, we have demonstrated that an implanted gelatin sponge initiates the formation of a granulation tissue that resembles a healing surgical wound. This can be used as a reproducible model to investigate tumour seeding in a peritoneal wound site. We have also identified several genes that are differentially expressed in early and intermediate granulation tissue, which may also be involved in tumour invasion and formation. Subsequent studies will be performed to examine the functional role of these genes on tumour cell invasion and growth into granulation tissue.

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