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The influence of tumour resection on angiostatin levels and tumour growth — an experimental study in tumour-bearing mice

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Abstract

The phenomenon of primary neoplasms inhibiting the growth of their metastatic lesions is thought to be related to endogenous angiogenesis inhibitors. The aim of this experiment was to investigate the influence of tumour resection on angiostatin levels and tumour growth using a tumour-bearing mouse model. A primary Lewis lung cancer tumour model was established in C57BL/6 mice and these mice were divided into two groups 10 days after the tumour cells were inoculated. In the surgical resection group (S group) the tumour was resected, but in the control group (C group) a sham operation was performed. The level of angiostatin in the sera was analysed 5 days after the operation by western blotting. To observe tumour growth, four Lewis lung cancer models were established in these mice from both the S and C groups. An immunohistochemical analysis of the tumour tissues was conducted to estimate the proliferation and apoptotic rates of the tumour cells, as well as the amount of neoangiogenesis in the tumours. Angiostatin was observed in the tumour-bearing mice, but disappeared within 5 days after the tumour had been resected. Increased tumour growth was observed in all of the tumour models in the S group compared with the C group and the differences were significant. A significantly higher intratumour vessel density and proliferation cell index, but a significantly lower apoptotic index were also found in the S group compared with the C group. These findings demonstrated that angiostatin was generated directly from the tumour tissue. Furthermore, tumour resection accelerates the growth of other tumours and this is probably related to multiple factors including increased neoangiogenesis, increased tumour cell proliferation, and decreased apoptosis.

Keywords: Angiostatin; Tumour resection; Inhibition; Angiogenesis; Apoptosis; Proliferation

1. Introduction

The inhibition of growth of their metastatic lesions by primary neoplasms has been explained by a specific immune response [1,2], the depletion of nutrients [3,4], and the production of some antimitotic factors derived from the primary tumour [5,6]. O'Reilly found two types of endogenous angiogenesis inhibitors, angiostatin and endostatin, which were related to the inhibition of metastatic growth by the primary neoplasms [7,8]. Some experimental studies examining the effects of angiostatin or endostatin administration have resulted in substantial inhibition of the tumour growth by inhibiting endothelial cell proliferation and neo-angiogenesis or by increasing tumour cell apoptosis [9,10]. It has observed clinically that the metastatic lesions grow rapidly if the primary tumour is resected.

In this study, we investigated the influence of surgical tumour resection in the generation of angiostatin and the growth of different tumour models, using the Lewis lung cancer tumour-bearing model in mice, and tried to examine the mechanisms involved.

2. Materials and methods

2.1. Animal and cell line

Six- to 8-week-old C57BL/6 male mice were used for all the experiments, which were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. Animals were bred in a standard laboratory and allowed free access to food and water in a temperature-controlled environment with a 12-h light and dark cycle.

The cell line of Lewis lung cancer (3LL) was kindly supplied by the Pharmaceutical Institute, School of Medicine, Keio University, Japan. Cells were main-

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tained in culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Tumour cells were inoculated subcutaneously (s.c.) into the mice and the tumour was freshly harvested when it had grown to approximately 1.5 cm in diameter. A single tumour cell suspension was prepared in phosphate-buffered saline (PBS) by the passage of fresh tumour tissue through a sequential series of 18-, 22-, 27- and 30-gauge needles. The 3LL cells (1×10^6) suspended in 0.1 ml of PBS were injected s.c. into the dorsa of mice. Animals were randomised into two groups 10 days after tumour cell implantation. In the surgical resection (S-group), mice underwent surgical resection of the dorsal tumour under general anaesthaesia; but in the control group (C-group), mice were anaesthetised and a sham operation was performed.

2.2. Western blotting analysis of the production of angiostatin

To investigate whether angiostatin is produced from the tumour and released into the sera of tumour-bearing mice, five mice from each group were killed 5 days after the operation. The sera were collected and filtered with a 0.22 μm filter. Sera samples containing 150 μg protein were electrophoresed under non-reducing conditions on 12% polyacrylamide gels in Tris-glycine running buffer and electrotransferred to a 0.45 μm polyvinylene difluoride membrane. The membrane was then blocked for 60 min with blocking buffer (5% milk in Tris-buffer saline, pH 7.5), and then incubated overnight with a 1:1000 dilution of a polyclonal antibody to the kringle 1-3 fragment of human plasminogen (Biogenesis Ltd, UK). After being washed, the membrane was incubated for 60 min with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Amersham) at a 1:1000 dilution and visualised using a western blotting detection kit (Amersham, Piscataway, USA).

2.3. Tumour growth analysis using several tumour models

To investigate the influence of tumour resection on the growth of different tumour models, four second tumour models were established in these mice both in the S ($n=20$) and the C groups ($n=20$), by a secondary inoculation of 3LL cells as follows.

For the allotopia-transplantation primary hypodermic tumour model, 1×10^6 3LL cells suspended in 0.1 ml of PBS were injected s.c. into the secondary site of dorsa, about 2 cm in distance from the first tumour mass. Animals were killed at 14 days after the secondary tumour inoculation, and the secondary tumour nodules were removed and weighed.

For the orthotopia-transplantation primary lung tumour model, a small incision through the skin and muscle was made in the left chest, but no thoractomy was performed. Next, 1×10^6 3LL cells suspended in 10 μl of PBS were inoculated into the left lung parenchyma by direct injection with a 27-gauge needle under an operative microscope, and the incision was closed with simple interrupted sutures. Animals were killed at 10 days after cell injection as about 30% of the mice died at 14 days. The second implanted tumour nodule and the left lung were removed and weighed.

For the experimental lung metastatic tumour model, 1×10^6 3LL cells suspended in 0.5 ml of PBS were injected intravenously (i.v.) into the tail vein. Animals were killed at 14 days after cell injection. Lungs were removed and the tumour nodes in the surface of lungs were counted under the microscope.

For a spontaneous lung metastatic tumour model, an i.v. injection of only 0.5 ml of PBS was given via the tail vein. Animals were killed 14 days after injection. The lungs were removed and weighed.

2.4. Immunohistochemical analysis

All the tumour and lung tissues were embedded in paraffin after being fixed overnight with 4% formaldehyde in PBS. Tissue sections, 5 μm thick, were used for immunohistochemical analysis according to the procedures described below. All tissue sections were pretreated with protein-digesting enzyme (5 mg/ml, Oncor, Gaithersburg, MD, USA) at room temperature for 20 min. An additional pretreatment was performed by boiling the tissue sections in 10 mM citrate buffer for 15 min before staining with monoclonal antibody against the von Willebrand factor (vWF) (1:20 dilution, Dako, Denmark). A PC-10 monoclonal antibody (1:50 dilution, Dako) was used to stain the proliferation cell nuclear antigen (PCNA). Positive stainings were detected by sequentially incubating with a biotin-labelled secondary antibody and the avidin-biotin peroxidase complex (Dako) according to the protocol of the manufacturer. The sections were washed three times with PBS before each incubation, then counterstained with Gill's haematoxylin and mounted in Gelmount. Apoptotic cells were stained by the Tdt-mediated dUTP-biotin nick-end labelling (TUNEL) method using the Apop-Tag Peroxidase Kit (Oncor) according to the protocol of the manufacturer. The counterstain was performed with 1% methyl green (Sigma, Louis, USA). The proliferation cell index and apoptotic index were defined as the percentages of positively stained cells per 100 cells, and at least 2000 cells in random fields were scored under the microscope at 200× magnification. Intratumoral microvessel density was counted using the method described by Weidner [11]. After the vWF-positive hot spots were localised at low magnification, microvessels in the hot spots were counted under a microscope with a 200-fold magnification. A separate positive-staining single cell or cell cluster was counted as one microvessel, and the

mean of microvessel counts in a 200-fold magnification field were calculated for the statistical analysis.

2.5. Statistical analysis

Statistical analysis was performed by the unpaired *t*-test. Data are presented as means ± standard deviation (S.D.) and significance was defined as $P < 0.05$.

3. Results

3.1. Angiostatin is generated directly from the tumour tissue and released into the serum

In the C group, the sera from the tumour-bearing mice showed a positive immunoreactive band at approximately 38 kD to a polyclonal antibody to the kringle 1-3 fragment of human plasminogen (Fig. 1). The positive band indicated that angiostatin was contained in the sera of tumour-bearing mice because angiostatin is a cleavage product of approximately 38 kD (kringle 1-4) of plasminogen and the polyclonal antibody used reacts specifically with the cleavage products of plasminogen. However, the positive immunoreactive band disappeared in the sera of the S group mice within 5 days after the dorsal tumour had been resected.

3.2. Acceleration of the growth of the various tumour types after tumour resection

The growth of various tumour types in mice in the S and in the C groups is shown in Fig. 2. In the allotopia-transplantation primary hypodermic tumour model, the second inoculated tumour was significantly bigger in the mice of the S group than of the C group, at 1064 ± 202 mg versus 412 ± 169 mg in tumour weight, respectively ($P = 0.011$). In the orthotopia-transplantation primary lung tumour model, the second inoculated tumour nodule in the left lung was also significantly bigger in the S group mice than in the C group mice, at 190 ± 85 mg versus 74 ± 22 mg in left lung weight, respectively ($P = 0.022$). In the experimental lung metastatic tumour model, a significant increase in the number of lung tumour nodules, but not in the weight of the lungs (data not shown) was observed in the S group mice compared with the C group mice, at 12 ± 3 versus 6.6 ± 1.9 tumour nodules in the surface of lungs, respectively ($P = 0.0086$). The lung weights were also significantly higher in the S group mice than in the C group mice in the spontaneous lung metastatic tumour model, at 1020 ± 317 mg versus 384 ± 143 mg in lungs weight, respectively ($P = 0.025$).

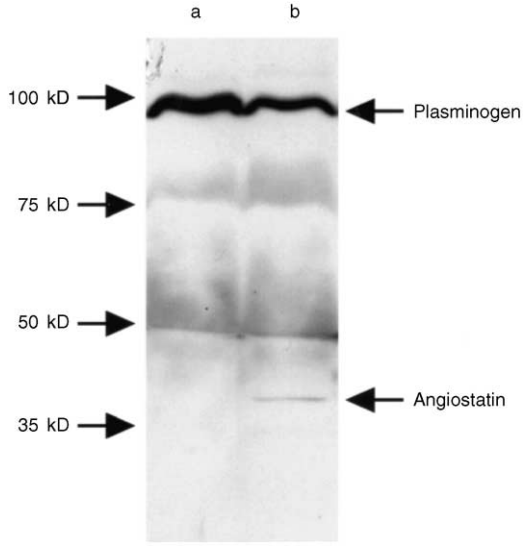

Fig. 1. Western blot analysis for angiostatin in the sera of mice. Lane a, no positive band was found in the sera of mice 5 days after surgical resection of the tumour (S group). Lane b, a distinct band was found of approximately 38 kD in the sera of the tumour-bearing mice (C group).

3.3. Increased neo-angiogenesis and tumour cell proliferation, decreased tumour cell apoptosis after tumour resection

The microvessel density, proliferation cell index, and apoptotic index in the various tumour models are shown in Figs. 3–5. We observed that the microvessel density in the second inoculated tumour tissues were significantly increased in the S group mice compared with that in the C group mice, according to the immunostaining analysis with monoclonal antibody against the von Willebrand factor (Fig. 3). We also found a significantly higher proliferation cell index of tumour cells in the S group mice than in the C group mice, in immunostaining experiments with the PC-10 monoclonal antibody (Fig. 4). The apoptotic index of tumour cells was decreased significantly in the S group mice compared with the C group mice, in immunostaining analysis using the TUNEL method (Fig. 5). No significant differences in microvessel density, proliferation cell index, or apoptotic index were found among the different tumour models.

4. Discussion

In this study, a distinct band representing angiostatin was observed in the sera of the 3LL tumour-bearing mice by western blotting analysis. However, the positive band disappeared within 5 days after the removal of the primary tumour. Although a very small amount of angiostatin may have been present in the sera of the mice from which tumours had been removed that was below the detection level of the western blotting analysis given that some studies have purified angiostatin from

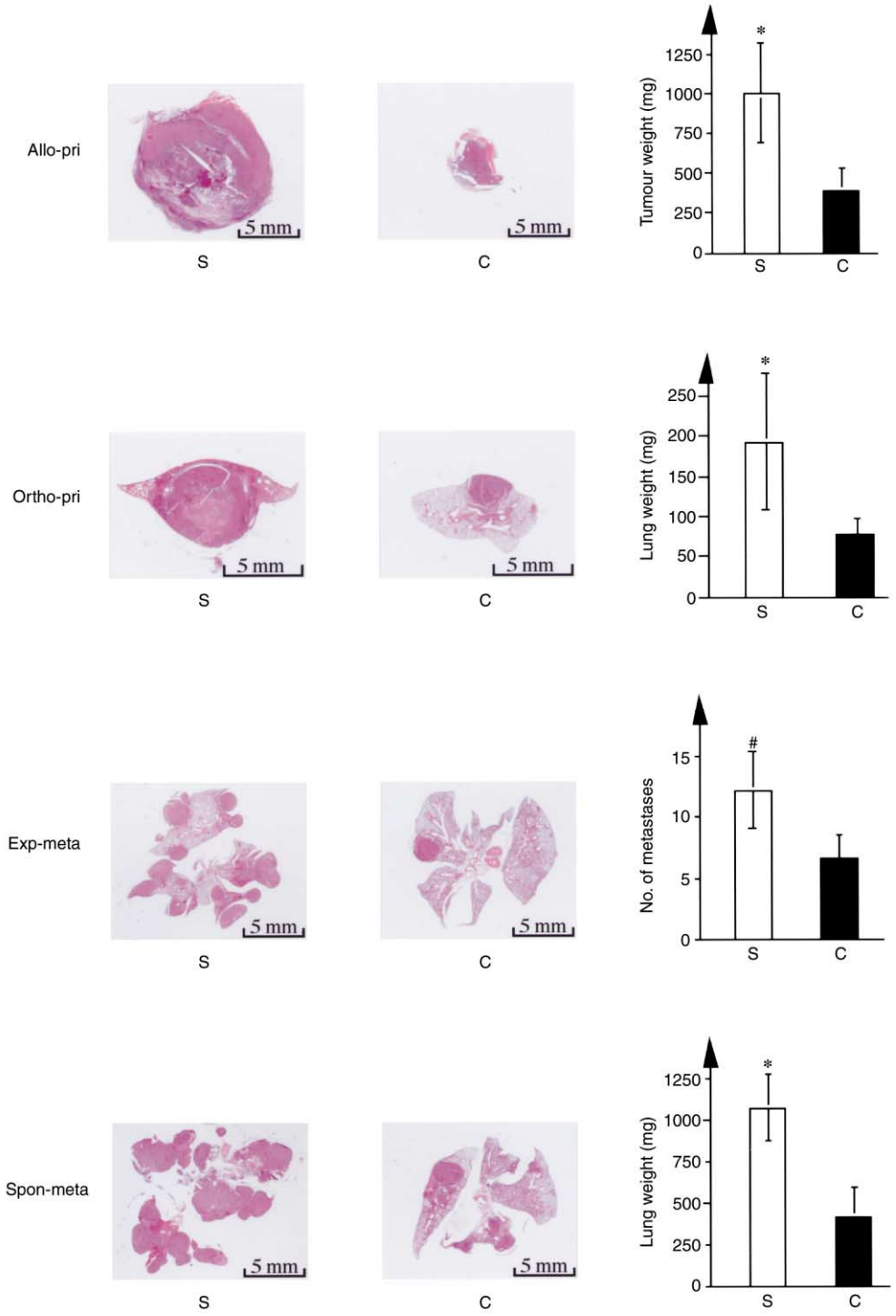

Fig. 2. The tumour growth pattern of several tumour models. Representative haematoxylin and eosin stained sections from mice in the S group (left panels) and in the C group (middle panels). Statistical analysis (right panels) showed that the growth of the second tumours was more significant in the mice of the S group compared with the mice of the C group in several tumour models. Allo-Pri, allotopia-transplantation primary hypodermic tumour model; Ortho-Pri, orthotopia-transplantation primary lung tumour model; Exp-Meta, experimental lung metastatic tumour model; Spon-Meta, spontaneous lung metastatic tumour model; S, S group; C, C group. *$P<0.05$, #$P<0.01$ versus C group.

normal human plasma [12]. Similar to the findings of O'Reilly and colleagues [7], our results provided direct proof that angiostatin was generated by tumour tissues in tumour-bearing mice, and that most the angiostatin was metabolised rapidly.

Sim and colleagues directly administered recombinant angiostatin protein to primary and pulmonary metastatic Lewis lung cancers, and observed a stronger tumour inhibition of the metastatic lesions than the primary ones; although less angiostatin was adminis-

tered to the primary tumours [13]. In this study, we observed that the growth of various tumour models was inhibited by a primary tumour lesion in the tumour-bearing mice, but was accelerated after these tumour lesions were resected surgically. However, the inhibition of tumour growth in the C group mice did not differ in the different tumour models. This result shows that the pattern of tumour growth inhibition by the tumour mass was different from the direct administration of angiostatin. The different tumour inhibitive patterns provided indirect evidence that the tumour inhibition by a primary tumour mass was not only derived from the inhibition of neo-angiogenesis as a result of the endogenous angiogenic inhibitor of angiostatin, but also from other factors, such as the specific immune response or the depletion of nutrients.

It has been found that micrometastases remain dormant by regulation of the balance between proliferation and apoptosis of tumour cells when a primary tumour mass is present or anti-angiogenic treatment is given [10,14,15]. The antitumour effect has also been demonstrated to be related to an inhibition of neo-angiogenesis and an increase in tumour cell apoptosis, but with no significant change in the proliferation of the tumour cells. We observed a lower intratumour density in the microvessels and a higher apoptotic index in the tumour-bearing mice than in mice from which tumours had been removed. The lower rate of neo-angiogenesis in the tumour-bearing mice was possibly derived, at least in part, from the angiostatin generated by the primary tumour, whereas the higher apoptotic index possibly resulted partly from the low amount of neo-angiogenesis and the poor nutrient micro-environment of the tumours in the tumour-bearing mice.

In contrast to other investigations [10,14,15], we have also found a significantly lower proliferation cell index in the tumour-bearing mice. This result indicates that tumour inhibition by another tumour mass might also be related to a decrease in tumour cell proliferation. The low tumour cell proliferating activity in the tumour-bearing mice may be derived from some antimitotic effects of the primary tumour, or from stimulation of growth factors after the removal of the tumour [5,6]. A low cell proliferating index would be induced by a deprivation in nutrition, and increased by a good nutritional supply [16,17]. In fact, it has also been frequently observed in clinical trials that metastatic lesions grow rapidly in patients given nutritional supplements. This finding suggests that nutritional depletion may be rela-

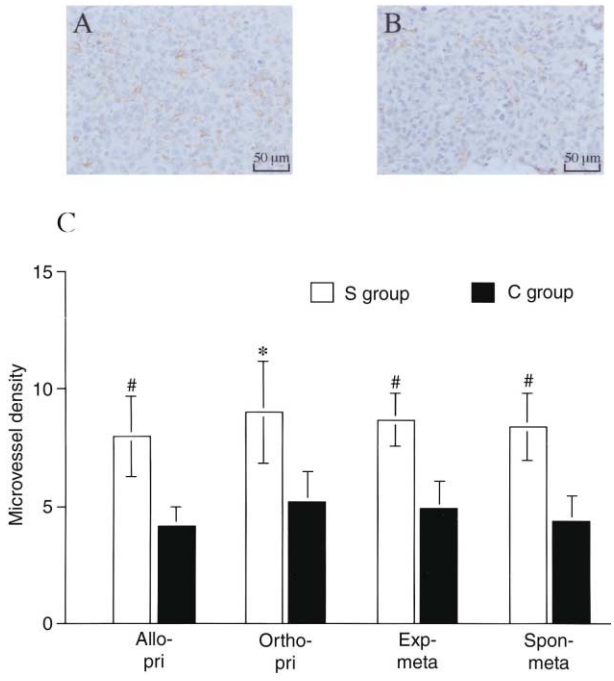

Fig. 3. Microvessel density in several tumour models. Representative sections of immunohistochemical staining with von Willebrand factor in the mice of the S group (A) and the C group (B). Statistical analysis (C) showed that significantly more microvessels were observed in the tumours of the S group than in the C group in several tumour models. Allo-Pri, allotopia-transplantation primary hypodermic tumour model; Ortho-Pri, orthotopia-transplantation primary lung tumour model; Exp-Meta, experimental lung metastatic tumour model; Spon-Meta, spontaneous lung metastatic tumour model. $*P<0.05$, $\#P<0.01$ versus C group.

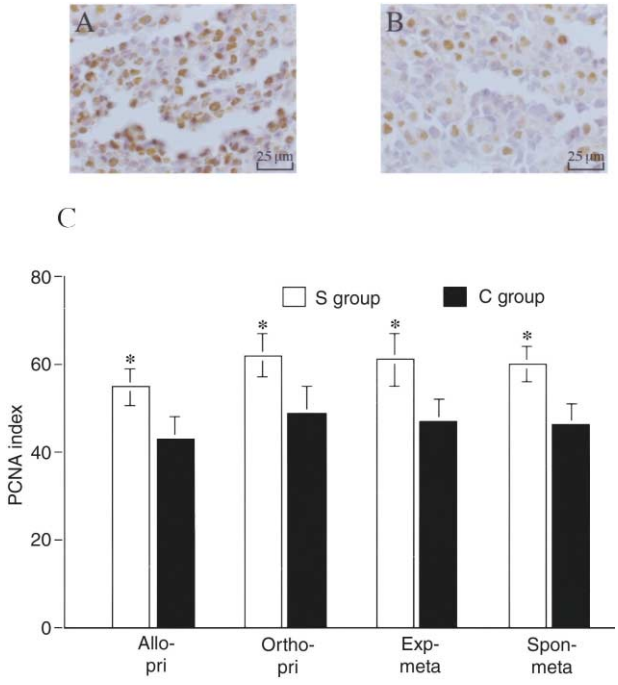

Fig. 4. The proliferating cell nuclear antigen (PCNA) index in several tumour models. Representative sections of immunohistochemical staining with PCNA in the mice of the S group (A) and the C group (B). Statistical analysis (C) showed that the PCNA index in the S group was significantly higher than in the C group in the various tumour models. Allo-Pri, allotopia-transplantation primary hypodermic tumour model; Ortho-Pri, orthotopia-transplantation primary lung tumour model; Exp-Meta, experimental lung metastatic tumour model; Spon-Meta, spontaneous lung metastatic tumour model. $*P<0.05$ versus C group.

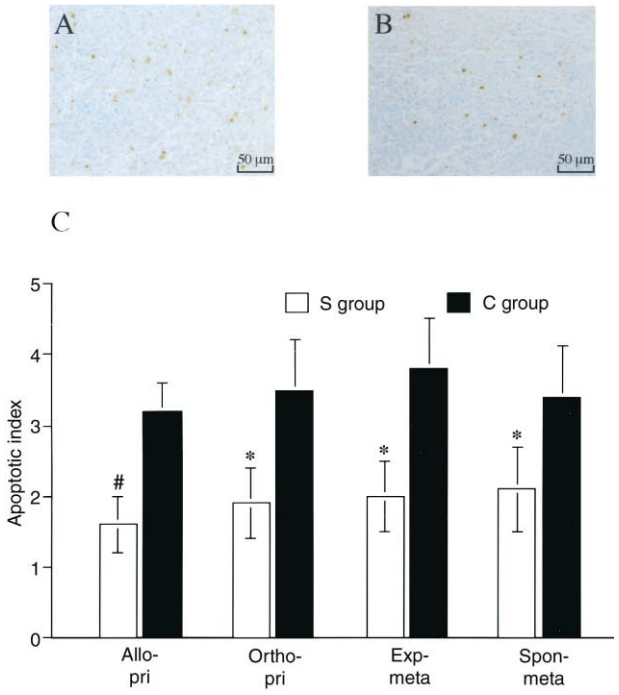

Fig. 5. The apoptotic index in several tumour models. Representative sections of immunohistochemical staining using the Tdt-mediated dUTP biolin nick-end labelling (TUNEL) method in mice of the S group (A) and the C group (B). Statistical analysis (C) showed that the apoptotic index in the S group was significantly higher than in the C group in the various tumour models. Allo-Pri, allotopia-transplantation primary hypodermic tumour model; Ortho-Pri, orthotopia-transplantation primary lung tumour model; Exp-Meta, experimental lung metastatic tumour model; Spon-Meta, spontaneous lung metastatic tumour model. $*P<0.05$, $\#P<0.01$ versus C group.

ted to the growth inhibition of the metastatic lesions by their primary tumour. Thus, surgical resection of the primary tumour would remove this inhibitory effect on tumour growth by their primary lesions or other tumours.

In conclusion, our experiments demonstrated that angiostatin was directly generated by the tumour tissue and disappeared rapidly following tumour resection. The primary tumour inhibited the growth of the second tumour lesions, but this inhibition was not correlated with either the organs or the origin of the tumour. The inhibitory effect on second tumour growth is removed following resection of the primary tumour, as this increases neo-angiogenesis, accelerates tumour cell proliferation, and decreases the apoptotic rate of the tumour cells.

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