The effect of subcutaneous tumour implantation in a murine lung tumour model

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Abstract

Objectives: It was hypothesized that if tumour were implanted subcutaneously within a Millipore Chamber (MPC), then this would result in an ‘anti-tumour’ immune response. Such an approach could have potential as an adjuvant tumour therapy when combined with surgical resection. A murine lung tumour model was used to test this hypothesis. Methods: Lung tumours were induced in 245 syngeneic mice by intraperitoneal 4-[methylnitrosamino]-1-[3 pyridyl]-1-butanone. In addition, MPCs were implanted containing either normal lung (Group A) or lung tumour (Group B). Group C had no implanted MPCs. These animals were sacrificed between 1 and 8 weeks following implantation and the stage of lung tumour development as assessed by the surface tumour count (STC) of their left lungs was compared between the different groups. The presence of CD4⁺ and CD8⁺ T cells in the local reactions surrounding the implanted chambers was also compared between Groups A and B at 1 week post-implantation. Results: At 1 week, the STC was significantly lower in Group B (2.4 ± 0.6) than in both Groups A (4.7 ± 0.6) and C (4.9 ± 0.9; P = 0.02). In addition, at 1 week, there was a significantly greater proportion (%) of CD4⁺ cells in the local reactions of Group B (52 ± 3) than in Group A (35 ± 3; P = 0.001). Between 2 and 8 weeks post-implantation, there were no further significant differences in tumour development between the groups. Conclusions: Although the findings of an ‘early’ response were consistent with the hypothesized benefit of tumour implantation within MPCs, the later results have not confirmed its potential as an adjuvant therapy.

Keywords: Immunotherapy; Surgery; Lung neoplasms; Models; Biological; Mice

1. Introduction

As with many tumour types, the only potential cure for lung carcinoma is surgical resection. However, even for early stages of the disease, the 5-year recurrence free survival rate for non-small cell lung carcinoma is only 40–70% [1]. Malignant cells remaining following resection cause recurrent disease. It is possible that the immune system, which is known to contribute to tumour defence [2], would be able to eliminate these remaining cells if they were relatively few. However, surgical resection is a ‘two-edged sword’. Whilst it is the only effective means for eliminating lung tumour, it also impairs the immune system that might otherwise eliminate residual cells. Surgery adversely affects many components of the immune response and has been demonstrated to enhance tumour spread both in animal models [3] and in clinical practice [4]. How tumours may be resected without at the same time causing an immunosuppressive effect that allows recurrences to develop remains an unresolved question.

A proposed solution arose from a study on the effect of subcutaneous implantation of lung in rats [5]. It was noted that when lung was implanted within Millipore Chambers (MPCs) into an allogeneic as compared with a syngeneic host, then a prolific immune response developed in the tissues surrounding the implanted chamber. It was inferred that antigens released from necrosing lung within the chamber passed through the pores of the Millipore Filters to interact with the local tissue. As a consequence, it was hypothesized that if lung tumour were similarly implanted then tumour antigen would be released into the local tissues causing an immune reaction, but at the same time the chamber would prevent local spread of the implanted tumour. It was therefore suggested that following surgical resection for lung carcinoma, if a segment of the tumour were to be re-implanted subcutaneously into that patient within a MPC, then it would augment the anti-tumour immune response and thereby reduce the likelihood of recurrent disease. A study using a murine lung tumour model was undertaken in...
order to assess whether such an intervention would be likely to have an anti-tumour effect.

2. Materials and methods

2.1. Tumour induction

Lung tumours were induced in mice using intraperitoneal 4-[methylnitrosamino]-1-[3 pyridyl]-1-butanone (NNK). NNK induces proliferation of type II cells along the alveolar septae and these hyperplastic lesions develop into adenomas and carcinoma [6]. Ten micromoles of NNK (Lancaster Synthesis Ltd., Morecambe, Lancashire, UK) in 0.1 ml of normal saline were injected intraperitoneally into each of 245 female, syngeneic A/J mice, aged 6–9 weeks and maintained on an AIN 76A diet (Special Diet Services Ltd., Witham, UK). The average number of tumours induced 26 weeks post-injection has been reported as $5 \pm 1$ [7].

2.2. MPC implantation

Sixteen weeks following NNK injection (to allow time for adequate tumour development), MPCs containing either lung tumour or normal lung were constructed for implantation into the different experimental groups. The components of a MPC consisted of a plastic holding chamber, two fixation discs and two 8 μm pore size Millipore Filters of 13-mm diameter (Millipore UK Ltd., Watford, UK). Lung tumours or normal lungs harvested from sacrificed mice who had either received or not received intraperitoneal NNK, respectively were placed in the holding chambers which were then closed by sealing a Millipore Filter at both ends using the fixation discs (Fig. 1).Recipient syngeneic A/J mice (all part of the original 245 that had received NNK) were anaesthetized using an intraperitoneal Hypnorm/Midazolam/water mixture. A dorsal longitudinal incision was made and a subcutaneous plane developed into which was inserted a chamber containing either normal lung or lung carcinoma and the incision closed with a subcutaneous suture. The experimental groups so formed were as follows:

- Group A ($n = 66$), chambers containing normal lung implanted into mice with induced lung tumours.
- Group B ($n = 76$), chambers containing lung tumours implanted into mice with induced lung tumours.
- Group C ($n = 67$), no chambers implanted into mice with induced lung tumours.

2.3. Experimental measurements

At between 1 and 8 weeks post-implantation, mice were sacrificed as follows:

- 1 week: Group A (10); Group B (10); Group C (10).
- 2 weeks: Group A (12); Group B (12); Group C (12).
- 4 weeks: Group A (12); Group B (12); Group C (12).
- 6 weeks: Group A (9); Group B (12); Group C (13).
- 8 weeks: Group A (8); Group B (14); Group C (17).

(The difference between the number of subjects and the original number of 245 injected with NNK was due to fatalities in the experimental groups and to those sacrificed to provide donor lung).

The stage of tumour development was assessed in all the experimental subjects. The left lungs (predominantly composed of the cephalic lobe) were all photographed (medial and lateral surfaces) in a standard manner to allow comparative measurements. For each subject, the total number of visible tumours was recorded as the surface tumour count (STC).

An immunological assessment of the local reactions surrounding the implanted chambers in Groups A and B was made at 1 week post-implantation. The local reactions were harvested and cellular preparations made by ‘grinding’ them over a 40 μm filter. Following centrifugation and removal of red blood cells by Tris Ammonium Chloride, the samples were mixed with 10 μL of fluorescent labelled monoclonal antibodies for CD4 and CD8 (Caltag Laboratories, Burlingame, CA) and the presence of labelled cells was measured by fluorescence activated cell sorting (FACS) analysis using a Becton Dickinson scanner.

2.4. Statistical analysis

The STCs of the lungs at 1–8 weeks post-implantation and the numbers (%) and degree of expression (mean fluorescence index; mfi) of CD4$^+$ and CD8$^+$ cells in the local reactions at 1 week post-implantation were compared.

![Fig. 1. A Millipore Chamber.](image)
between the different experimental groups using the Student’s t-test. A statistical software package was used (Stat View, Abacus Concepts, Berkley, CA). Data are expressed as mean ± SEM.

2.5. Animal care

All the animals received humane care in compliance with the European Convention on Animal Care. The experimental work was reviewed and licensed by the United Kingdom Home Office and subject to regular inspection.

3. Results

A comparison between the measured STCs in the different experimental groups is shown in Table 1. At 1 week post-implantation, the STC was significantly less in Group B (2.4 ± 0.6) than in both Groups A (4.7 ± 0.8) and C (4.9 ± 0.6; *P = 0.02). Representative examples of these lungs are shown in Fig. 2. At later implantation times, no further significant differences of tumour development were found between the groups.

A comparison of the proportions (%) and expression (mfi) of CD4+ and CD8+ T cells in the local reactions surrounding the implanted chambers 1 week following implantation are shown in Table 2. There was a significantly greater proportion (%) of CD4+ cells in Group B (52 ± 3) than in Group A (34.7 ± 3; *P = 0.001). In addition, there was a significantly greater expression (mfi) of CD4+ cells in Group B (16.4 ± 0.3) than in Group A (14.4 ± 0.6; *P = 0.045). A similar comparison was not made at later implantation times.

Table 1
Stage of tumour development as assessed by STC in experimental groups A, B and C at 1–8 weeks post-implantation of MPCs

<table>
<thead>
<tr>
<th>Weeks P.I.*</th>
<th>Group</th>
<th>N</th>
<th>STC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>10</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>12</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>12</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>12</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>12</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>9</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>8</td>
<td>4.6 ± 1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>14</td>
<td>6.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>17</td>
<td>6.8 ± 0.8</td>
</tr>
</tbody>
</table>

* P.I., post-implantation.

Table 2
The proportion* and expression* of CD4+ and CD8+ cells in the local reaction surrounding implanted MPCs in Groups A and B 1 week following implantation

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 %</td>
<td>34.7 ± 3</td>
<td>52 ± 3; *P = 0.001</td>
</tr>
<tr>
<td>mfi</td>
<td>14.4 ± 0.6</td>
<td>16.4 ± 0.3; *P = 0.045</td>
</tr>
<tr>
<td>CD8 %</td>
<td>60.4 ± 6</td>
<td>54.1 ± 5</td>
</tr>
<tr>
<td>mfi</td>
<td>13.8 ± 0.4</td>
<td>13.7 ± 0.3</td>
</tr>
</tbody>
</table>

* Proportion (%); expression (mfi).

Fig. 2. Representative examples of the left lungs (medial and lateral surfaces) from Groups A, B and C showing the relative number of tumours at 1 week post-implantation.

4. Discussion

It has previously been demonstrated that the immune system plays a role in tumour defence [2]. Immunotherapy techniques that have been used for the treatment of lung carcinoma have included the inoculation of allogeneic [8] and autologous tumour cells [9]. In addition, adoptive immunotherapy, which makes use of cultured ‘effector’ cells, has been applied. This has included the use of lymphokine activated killer cells (LAKs) [10] and tumour infiltrating lymphocytes (TILs) [11]. However, whilst benefit has been demonstrated with some of these techniques, this has neither been consistent nor large enough to make any major clinical impact.

Conceptually, an immune response may be expected to be effective against tumour cells when they are relatively few. Unless it is combined with other treatments, it is unlikely to be effective against large tumour masses or where there is widespread disease. One situation where there are relatively
few tumour cells is following an attempted ‘curative’ surgical resection. Recurrences following such surgery originate from residual malignant cells. It might be expected that the patients’ innate immune response would have the potential to eliminate these. However, surgery itself may prevent this because of its tendency to depress the immune response. Reported effects have included decreased opsonic capacity, antigen presentation and numbers of CD4\(^+\), CD8\(^+\) and natural killer cells [12,13]. This surgically induced immunosuppression may explain the observation made both in animal models [3] and in clinical practice [4] that surgery may enhance tumour spread. Thus, a clinical situation where immunotherapy may be most effective in the treatment of malignant disease is following potentially ‘curative’ surgery both because of the relatively small number of remaining tumour cells and also to compensate for the depression of the ‘natural’ immune response caused by the surgery. This is not the situation for which most reports have described its application and this may partly explain their disappointing results.

The hypothesized approach to immunotherapy that this study has explored was developed with the intention of providing a means to enhance the immune response that could be easily and therefore routinely applied following surgical resection for lung carcinoma. The experimental observation upon which it was based was the ability of subcutaneously implanted rat lung within a MPC to induce a profound local immune response in an allogeneic recipient [5]. The ability of tissue to induce an immune reaction in another tissue from which it is separated by a filter implies that relevant antigens can pass through the filter. It is likely that such antigens originate from the enclosed non-vascularized tissue as it undergoes necrosis. This observation led to the suggestion that if tumour were to be similarly implanted within a MPC then released tumour antigens would induce an immune response in the surrounding tissues whilst the chamber itself would prevent local spread. The proposed clinical application was that following lung resection for carcinoma a segment of the resected tumour would be placed within a MPC and re-implanted subcutaneously into the same patient. In that situation, it was hypothesized that released tumour antigen would induce an anti-tumour immune response, which would compensate for surgically induced immunosuppression. If any remaining tumour cells were relatively few then this would contribute to their eradication.

This study was carried out to partly assess the validity of the hypothesized approach. The tumour model used was an approximation to the proposed application. Syngeneic mice were used in order to mimic the implantation of ‘self’ tumour. The model differed from the proposed application in that there were no tumour resections (and therefore no debulking) in the implanted animals. Also, the induced tumours in this model were predominantly adenomas and adenocarcinomas, which differs from the usual clinical spectrum. However, it was believed that the approximation was sufficient to examine some of the techniques underlying assumptions.

The results of this study were mixed. One week following implantation of the chambers there was significantly less tumour development as assessed by the STC in Group B than in either Groups A or C. In addition, at 1 week post-implantation, there was evidence for the induction of an immune response in the local reactions surrounding the implanted chambers in Group B. Group B was the active group that had chambers containing tumour implanted and Groups A and C were control groups. This was therefore consistent with the hypothesized approach having an anti-tumour effect secondary to an induced immune response. The involvement of CD4\(^+\) cells is consistent with tumour antigen being taken up by antigen presenting cells and represented on their surface together with MHC Class II molecules, which in turn would be expected to result in the activation of CD4\(^+\) T cells. Studies in human and murine systems have suggested a central role for CD4\(^+\) T cells in initiating, effecting and maintaining anti-tumour immunity [14].

However, the later observations do not support the likelihood that this effect is maintained. At no other implantation times (2–8 weeks) were significant differences seen in tumour development between the different experimental groups. The failure to demonstrate a prolonged effect may have a number of causes. The lung tumours within the chambers at 6–8 weeks post-implantation appeared amorphous with few if any nucleated cells. Thus, any effect would be likely to be a short-term one. Although antigens are released by necrosing tumours, these are not necessarily those that would induce an appropriate anti-tumour immune effect. It is also possible that any immune response induced in this model is too localized for a prolonged effect. To answer this will require further studies looking at cytokine release as well as the development of the local response beyond 1 week.

As regards the safety of the proposed technique, it was observed that in none of the experimental subjects was disruption of the chambers or local spread of tumour beyond the implanted chamber identified.

In summary, this study has demonstrated in an animal model that the technique of implanting tumour within a MPC into tumour bearing recipients appears to have an early anti-tumour effect and is safe. However, it failed to demonstrate a persisting anti-tumour effect and therefore has not provided convincing evidence that it would be effective in clinical practice. It remains speculative whether another tumour type or model would have been more likely to demonstrate such evidence.

Acknowledgements

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References