Uptake and Distribution of Specific and Control Monoclonal Antibodies in Subcutaneous Xenografts following Intratumor Injection

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ABSTRACT

Nude mice bearing s.c. xenografts of the human colon adenocarcinoma HT29 were given intratumor injections of a mixture of 125I-labeled specific antibody (AUAA) and 131I-labeled control antibody (HMFG1), or with the labels reversed.

After dissection at 1 and 4 h postadministration, both specific and control antibodies had 47-63% of the injected dose (% ID) in the tumor. By 24 h, the tumor contained 43 ± 11% ID of AUAA which persisted at all times, resulting in specificity indices greater than 20 by 5 days.

 Autoradiography of tumors removed 2 h postinjection of 125I-labeled AUAA or HMFG1 showed high levels of antibody at the injection site. At 48 h and 7 days postinjection, the specific antibody was bound to the surface of tumor cells in islands remote from the injection site, whereas the control antibody was found only in the stroma and blood vessels, or as diffuse nonspecific uptake.

These data indicate that intratumor injection of radiolabeled monoclonal antibodies may achieve high radiation doses in accessible tumors without systemic irradiation.

INTRODUCTION

Although radiolabeled monoclonal antibodies are being investigated for diagnosis and treatment of malignant disease (1, 2), antibodies administered i.v. for therapy have several drawbacks which limit the radiation dose delivered to the tumor. These include the high systemic radiation dose due to circulating antibody, the low absolute uptake by the tumor, typically 0.001-0.01% of the injected dose/g (3), and the catabolism of the antibody in vivo by enzymes in the liver and other normal organs. In addition, patients may develop antibodies against the murine monoclonal antibodies used for therapy (4), precluding the repeated treatments which may be necessary to achieve a tumoricidal dose of radiation.

Regional therapy is an alternative to i.v. injection in cases when the disease is confined to a body cavity, and may overcome some of these problems. In a xenograft model of i.p. tumors, we have shown the short term advantage of i.p. over i.v. administration of radiolabeled antibodies to be approximately 50-fold (5). Clinically, i.p. administration of radiolabeled antibodies has been used to treat patients with cutaneous metastatic melanoma, in the brain tumors and observed objective responses; and malignant breast cancer, which can be treated by radioactive wires and implants (9), direct intratumor injection of radiolabeled monoclonal antibodies may be advantageous. Intratumor injection of tumor necrosis factor for the therapy of human tumor xenografts implanted in nude mice has been shown to be far more effective than the same dose given i.p. (10). Clinically, glioblastoma has been treated by intratumor injection of autologous lymphocytes plus human lymphoblastoid interferon, and morphological studies showed that the injected lymphocytes remained within the tumor (11). LAK3 cells treated with bispecific antibody have been shown to be effective when given locally after tumor debulking in patients with malignant glioma (12).

The aims of the work presented here were to improve the tumor uptake of radiolabeled monoclonal antibody by direct intratumor injection, and to study the diffusion through the tumor at the cellular level. In addition, the uptake and distribution of a specific and a control antibody were compared, to determine to what extent antibody specificity is important via this route of administration. To the best of our knowledge, this is the first study of intratumor injection of radiolabeled monoclonal antibodies.

MATERIALS AND METHODS

Tumors. HT29 was established in 1964 (14) from the primary tumor in a female patient with adenocarcinoma of the colon. The cell morphology is epithelioid. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland) in 175-cm² Falcon tissue culture flasks (Becton Dickinson, Lincoln Park, NJ). When confluent, cells were harvested by using 0.06% trypsin in 0.02% EDTA solution, washed with medium, and resuspended at a concentration of 5 x 10⁶ cells/ml in tissue culture medium.

Tumor xenografts were initiated in the right flank of male nude mice of mixed genetic background (ICRF Animal Breeding Unit, South Mimms, Hertfordshire, United Kingdom) by a s.c. injection of 5 x 10⁶ cells in a volume of 100 μl. For some studies, bilateral tumors were implanted into groups of mice. Animals were used for experiment 4 to 5 weeks later when the tumor diameter was 7-10 mm.

Antibodies. AUAA was raised against a human colon adenocarcinoma cell line (15). It is an IgG1 immunoglobulin directed against a M, 35,000 protein associated with the majority of human gastrointestinal, ovarian, and breast carcinomas, as well as normal proliferating epithelial cells (16). It reacts strongly with a cell surface antigen on HT29 cells, both in vitro and in vivo.

HMFG1, also IgG1, was raised by Taylor-Papadimitriou (17) against delipidated human milk fat globule. It recognizes a carbohydrate determinant on a high molecular weight glycoprotein (M, > 400,000) normally produced by lactating human mammary cells, but was also found on many carcinomas (18). It does not bind to HT29 cells and was used as the control antibody.

Antibody Biodistribution. Antibodies were labeled with 125I or 131I (IMS30 and IBS30, respectively, Amersham International, Amersham, 2

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3251
Buckinghamshire, United Kingdom) to a specific activity of approximately 2 μCl/μg by using the Iodo-Gen method (19). The reaction mixture was purified by centrifugation through a 1-ml Sephadex G-50 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in order to minimize the total volume of iodinated antibody (20).

Five μg of a mixture of the two antibodies, each labeled with a different isotope, were injected into the tumor in a total volume of 5 μl, using a 27G butterfly and a 100-μl Hamilton microsyringe (Anachem, Luton, Bedfordshire, United Kingdom). All antibody injections were performed within 3 hours of radiolabeling. At various times after antibody injection, ranging from 1 h to 18 days, groups of at least 4 mice were performed within 3 hours of radiolabeling. At various times after injection, the tumors which had also received an intratumor injection of 125I-labeled AUA1 i.v., in addition to an antibody injection, ranging from 1 h to 18 days, groups of at least 4 mice were dissected and the activity in tumor and normal organs was determined by gamma counting. In a separate study, mice bearing bilateral tumors received 125I-labeled AUA1 i.v., in addition to an intratumor injection into one tumor of the same antibody labeled with 131I, in order to compare the two routes of administration directly, and to determine whether intratumor injection compromised the binding of systemically administered antibody.

 Autoradiography. Some groups of mice were given an injection of 5 μl of 125I-labeled specific or control antibody alone, and autoradiography was performed at 2 h, 48 h, and 7 days. Tumors were excised, fixed in Methacarn (21), processed through alcohols, paraffin embedded, and 5-μm sections were cut. Gross autoradiography was achieved by placing the slides in direct contact with autoradiographic film (Hyperfilm 12H, Amersham International) and exposing them for 8 days at 4°C in X-ray cassettes with intensifying screens. For microautoradiography, sections were deparaffinized with xylene, hydrated, and dipped in Ilford K5 emulsion at 45°C, then stored in light-proof boxes for 3 weeks at 4°C. Both types of autoradiograph were developed in Kodak D19 for 5 min. The microautoradiographs were then counterstained with Mayer’s hematein, dried, and mounted.

 Statistical Analysis. The statistical significance of the difference between means was determined by using the Student’s t test. P < 0.05 was considered to be significant.

RESULTS

Fig. 1 shows the percentage of the injected dose of antibody remaining in the whole tumor up to 18 days after injection. Each point represents the mean ± SD of between 6 and 17 mice for AUA1, and between 4 and 10 mice for HMFG1, including mice given injections of dual-label antibodies and those receiving only 125I-labeled antibody at the time of the autoradiograph experiments. Each tissue sample was counted together with triplicate samples of the injectate in order to correct for the decay of the isotope before gamma counting. The uptake of specific antibody is significantly higher than that of control at 48 h, and at all subsequent times. In the groups of mice which had untreated tumors on the contralateral flank, these tumors did not accumulate high levels of either antibody from the circulation following intratumor injection into the other tumor (<2% ID/g), indicating that the high levels of activity measured in the injected tumors were due to antibody remaining at the site of injection, rather than by diffusion of antibody into the blood and subsequent accumulation in tumor. There was no significant difference in the uptake of i.v. injected AUA1 between a tumor which had also received an intratumor injection of the antibody, and the uninjected contralateral tumor. For example, at 24 h after injection, the tumors which had also...
INTRATUMOR INJECTION OF ANTIBODIES

Fig. 2. Percentage of injected 131I activity due to specific, AUAI (●), or control, HMFG1 (○), antibody remaining in the tumor, and due to AUAI in the blood (△) up to 18 days after intratumor injection. The animal numbers per point and the errors are as in Fig. 1, from which this figure is derived.

Table 2 Dosimetry for intratumorally administered antibodies

<table>
<thead>
<tr>
<th>Isotope</th>
<th>131I-AUAI</th>
<th>131I-HMFG1</th>
<th>32P-AUAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotope  ( t_\beta ) (days)</td>
<td>8.0</td>
<td>8.0</td>
<td>14.3</td>
</tr>
<tr>
<td>( \beta_{\text{max}} ) energy (MeV)</td>
<td>0.6</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>( ZA_{\mu}/\text{cGy/µCi} )</td>
<td>0.4</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>AUC (µCi-h/µCi)</td>
<td>71.9</td>
<td>24.5</td>
<td>90.3</td>
</tr>
<tr>
<td>g·cGy/µCi</td>
<td>2.88</td>
<td>980</td>
<td>133.7</td>
</tr>
<tr>
<td>Dose (cGy) to 1-g tumor</td>
<td>2,880</td>
<td>13,370</td>
<td></td>
</tr>
</tbody>
</table>

The equilibrium absorbed dose to a tumor is given by:

\[
D_m = \frac{A}{m} \sum \Delta_t
\]

where \( A \) is the time integral of activity (the AUC); \( \Delta_t \) is the

been injected intratumorally contained 0.5 ± 0.2% ID/tumor (4.8 ± 1.8% ID/g) of the i.v. administered antibody, and the untreated tumors contained 0.4 ± 0.3% ID/tumor (5.4 ± 0.2% ID/g). This contrasts with 40.2 ± 16.2% ID/tumor (365 ± 142 ID/g) of the intratumorally injected antibody at this time. Similar results were found at other times from 1 h to 5 days.

The levels of activity in blood, tumor, and representative normal organs (kidney, liver, lung, and spleen) are shown in Table 1. The mean tumor weight at each time point is also given to indicate any effect of tumor growth on the antibody uptake/g, although HT29 is a relatively slow-growing tumor, with a volume-doubling time of around 2 weeks. The errors on the tumor antibody uptake/g are large, since the concentration is more variable than the absolute uptake per tumor following intratumor injection. Table 1 indicates that the blood levels of both antibodies are very similar 1 h after injection, due to leakage of the proteins into the blood vessels within the tumor tissue. In the case of AUAI, there follows a rapid drop in blood activity between 1 and 4 h, due to extravasation of the antibody, and then a slow clearance from the circulation with a half-life of 85 h. This is comparable with the i.v. injection of AUAI (5). The HMFG1 blood levels are much more variable since the antibody is retained in the tumor nonspecifically, and therefore continues to be removed throughout the time course of the experiment, at a rate dependent on the individual tumor vasculature. The uptake by normal organs is very low for AUAI, but higher for HMFG1, which is not retained in the tumor. At all times after 4 h, the normal organ uptake of HMFG1 is greater than that of AUAI, whereas the tumor level of AUAI is very much higher than that of the control antibody. These figures result in specificity indices (SI), defined as

of greater than 13 by 3 days postinjection, for all tissues studied (stomach, intestine, kidney, spleen, lung, liver, skin, heart, bone, muscle, thyroid), with a range of 13.6 (stomach) to 24.5 (heart). The SI continued to increase with time after 3 days, up to nearly 50 by 18 days postinjection, due mainly to the large difference in tumor uptake of the two antibodies.

Allowing for the decay of the isotope within the tumor, Fig. 2 is derived from the data in Fig. 1, assuming exponential decay with a physical half-life of 8 days for 131I-labeled antibodies, and shows the percentage of injected 131I activity remaining in the tumor due to AUAI and HMFG1, and in the blood due to AUAI. For reasons of clarity, error bars have not been included in Fig. 2, but the errors on the blood uptake are given in Table 1, and the percentage errors on the tumor are equal to those in Fig. 1. The AUC, computed by summing trapezia, for the specific antibody in tumor is 2.9-fold higher than that for the control antibody in tumor, and 11.4-fold higher than that for AUAI in blood, if both antibodies were labeled with 131I.

Fig. 3. HT29 xenograft sections showing moderately to poorly differentiated adenocarcinoma (a, H & E). Tumor cells bind AUAI on their surface (b, indirect immunoperoxidase stain); (bar, 25 µm).
equilibrium dose constant; and $m$ is the tumor mass. $\Delta\varepsilon$ is a factor dependent on the energy emitted per disintegration in the form of $\beta$-type radiation (22); in the case of $^{131}$I, this radiation is in the form of either $\beta$-particles or $\gamma$-rays. If the AUC is expressed in $\mu$Ci-h, then $\Sigma\Delta\varepsilon$ is $0.4$ CGy/$\mu$Ci-h for a 1-g tumor irradiated by $^{131}$I uniformly distributed within the tumor volume (23), taking into account only the contribution of the $\beta$-particles, since most of the photon energy would not be deposited in tumor tissue. Table 2 shows the doses which would be delivered to a 1-g tumor following intratumor injection of 100 $\mu$Ci of $^{131}$I-labeled specific or control antibody. Higher doses would be expected from an isotope with a longer half-life and a more energetic $\beta$-particle, since this would result in a larger AUC and higher equilibrium dose constant. The last column in Table 2 shows the theoretical dose which would be delivered by $^{32}$P-labeled specific antibody, assuming the uptake of antibody by tumor to be the same when labeled with this isotope. Studies have shown that the binding of antibody to tumor cells in vitro is not impaired by $^{32}$P-labeling (24), and since the uptake of antibody by tumor cells following intratumor administration is not affected by systemic catabolism, the binding in vivo by this route should be very similar to that found for $^{131}$I-labeled antibody.

Following i.v. injection, the tumor uptake of iodinated AUAI was found to be approximately one-tenth of that measured after intratumor injection at all time points studied. The AUC, and therefore the total tumor dose, would consequently be reduced by the same factor, resulting in a dose too low to be tumoricidal. Similarly, blood and normal organ radiation doses resulting from intratumor injection would be very low since only a small percentage of specific antibody enters the circulation (Table 1).

The HT29 xenografted tumors grew as moderately to poorly differentiated adenocarcinomas with some acinar formation (Fig. 3a); necrosis was uncommon. An indirect immunoperoxidase stain indicated that nearly all tumor cells are positive for AUAI (Fig. 3b), showing cell surface staining. Gross autoradiography of the tumor showed that, at 2 h after injection, the uptake of both antibodies is similar, being largely confined to the injection site (Fig. 4, a and b). By 7 days after injection, the majority of the control antibody has been cleared from the tumor, while the specific antibody is retained and has diffused to areas remote from the site of injection (Fig. 4, c and d). Fig. 5 shows the microautoradiography of sections of tumor at 2 and 48 h after antibody injection. In areas remote (3–4 mm) from the site of injection at 2 h (Fig. 5, a and b), there is some activity in the blood vessels, accounting for the uptake in blood of both antibodies immediately after injection (Table 1). At 48 h, AUAI is specifically bound to the surface of tumor cells adjacent to the injection site (Fig. 5c). In contrast, HMFG1 shows no binding to tumor cells, but is present in the blood vessels and stroma as a diffuse, nonspecific uptake throughout the section (Fig. 5d).
INTRODUCTION OF ANTIBODIES

Fig. 5. Microautoradiography of HT29 tumor sections cut from areas remote from the intratumor injection site at 2 h (a, b) and adjacent to the injection site at 48 h (c, d) after injection of 131I-labeled specific (a, c) or control (b, d) antibody. (bar, 25 μm).

DISCUSSION

The data presented here indicate that high tumor uptake of specific radiolabeled antibody can be achieved in a s.c. human tumor xenograft model, with very low normal organ activity. If similar results can be achieved clinically in accessible tumors such as prostate and breast cancer, then tumoricidal doses of radiation should be possible without systemic toxicity. Dykes et al. (25), using a mathematical model of antibody uptake by a tumor, have calculated that in order for a dose of 6000 cGy in 1 week to be given to a tumor, while limiting the whole-body dose to 200 cGy, then at least a 10-fold increase is necessary in tumor uptake over that which is currently achievable. Admittedly, this figure is for humans, and much higher uptake is observed in xenografts in nude mice following i.v. administration, typically 5–20% ID/g (5, 26). However, the difference is due mainly to the volume of the blood pool in relation to tumor size, which would not be relevant to the intratumor route of administration, since the antibody is not accumulated from the bloodstream, as the studies with animals bearing bilateral tumors indicate. The graphs in Figs. 1 and 2 show the percentage of the injected dose remaining in the whole tumor, as opposed to per g of tumor tissue. Since the tumors weighed approximately 0.1–0.4 g, these figures represent uptakes of greater than 100% ID/g for AUA1, demonstrating that intratumor injection has achieved a 10-fold increase in tumor uptake compared with i.v. administration in a mouse model. This improvement should be far higher in a human, due to the large blood volume through which an i.v. injected antibody is distributed. Intratumor injection of antibody is clearly able to achieve a far higher increase than that indicated by Dykes et al., who assume a tumor uptake of 0.005% ID/g.

The importance of specificity was demonstrated by Nitta et al. (12), who compared locally administered antibody-bound LAK cells with nontargeted LAK cells in glioma patients after tumor debulking. No recurrences were seen in the group receiving targeted therapy, whereas the overall survival (2 of 10) in the group receiving LAK cells alone was similar to that seen with conventional treatment. They concluded that local administration of LAK cells is more effective than systemic delivery, since it probably maximizes cell to cell contact between killer cells and tumor cells, thus reducing the number of cells required. Specificity is also important in the delivery of a dose of radiation to the tumor following intratumor injection of radiolabeled antibodies. The dose due to 131I-labeled specific antibody is nearly 3 times higher than that due to an irrelevant 131I-labeled antibody, which does not bind to tumor cells and remain in situ, even though injected directly into the tumor tissue.

Although limited to easily accessible tumors, intratumor administration of radiolabeled monoclonal antibodies for therapy is applicable to any tumor currently treated interstitially with radioactive wires or seeds. Barringer (27) was among the first to treat prostate cancer with radioactive implants by inserting radon-containing 18-gauge needles directly into the...
prostate. Since then, radioactive gold (198Au) has been implanted in the form of seeds and combined with external beam irradiation (28). Whitmore (29) was able to deliver a dose over 2 or 12 months to the prostate of between 6000 and 12000 cGy, using 125I seeds, and reported positive results, particularly in patients with low grade tumors. One disadvantage of interstitial therapy is the nonuniformity of dose, which can result in some parts of the tumor receiving a noncytotoxic level of radiation. The autoradiographs presented in this paper show that the antibody is able to diffuse away from the site of injection to more remote tumor islands, and so should result in a more uniform radiation dose within a tumor than that achieved with radioactive implants.

Table 2 shows the dose to a 1-g tumor from 100 μCi of 131I or 32P. For a 20-g tumor, which is more relevant to primary prostate cancer, the doses would be 1440 and 6680 cGy/MCi of injected activity for 131I- and 32P-labeled specific antibody, respectively. However, for a tumor of this size, the antibody would have to be injected into several sites to ensure a more uniform distribution of activity within the tumor mass. These dosimetric calculations indicate that a dose of radiation sufficient to eradicate a tumor should be possible, while keeping the dose to bone marrow and other normal organs within acceptable limits.

In conclusion, these studies demonstrate that intratumorally injected radiolabeled monoclonal antibody of appropriate specificity is retained at high levels in the tumor, whereas an irrelevant antibody is cleared much more rapidly. Intratumor antibody is able to diffuse through the tumor and bind to tumor cells remote from the site of injection, while blood and normal organ activity remains very low. These observations indicate that a high dose of radiation may be given by radiolabeled antibody injected into an accessible tumor, without the systemic toxicity found with other routes of administration, particularly if a β emitter with a moderately long half-life is used. It is possible to label antibodies with 32P (βmax = 1.7 MeV; t½ = 14.3 days) via a short peptide (23), and studies on the therapy of s.c. xenografts with 32P-labeled antibodies are currently under way in our laboratory.

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