Angiogenesis in the Hollow Fiber Tumor Model Influences Drug Delivery to Tumor Cells: Implications for Anticancer Drug Screening Programs

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Abstract

The National Cancer Institute uses the hollow fiber assay as part of its screening program for anticancer drug discovery. Angiogenesis to hollow fibers implanted s.c. has not been reported, thereby raising concerns about the efficiency of drug delivery and its subsequent effects on chemosensitivity. By extending postimplantation times beyond the 6-day period presently used, extensive vascular networks develop, resulting in both increased delivery and chemosensitivity to doxorubicin. This study suggests that present protocols used to evaluate compounds may produce false negative results, and additional studies to determine the predictive value of the assay are required.

Introduction

In the late 1980s, the Developmental Therapeutics Program of the NCI3 radically changed its approach to the large-scale, random screening of compounds for anticancer activity (1). Instead of using xenografts or syngeneic rodent tumor models as a front line screen, all compounds submitted to the NCI are evaluated against a panel of well-characterized human tumor cell lines. Compounds are selected for further evaluation if they possess disease-specific activity in vitro, are COMPARE negative (i.e., have chemosensitivity fingerprints that differ from standard agents), or if activity in vitro is correlated with the expression of specific molecular targets in the cell line panel (1–4). It was envisaged that the primary in vitro screen would have such discriminative powers that only a few agents would need to be evaluated against human tumor xenografts. This, however, has not been the case (5), thereby creating a backlog of compounds awaiting evaluation against xenografts. Because of the cost associated with the use of xenografts to evaluate large numbers of compounds, the NCI developed and introduced an intermediate screen through which compounds must pass before full evaluation in xenograft tumor models (6). The hollow fiber assay (7, 8) used by the NCI involves the short-term in vitro culture (1 day) of tumor cells inside biocompatible, polyvinylidene fluoride fibers, followed by implantation into mice at two anatomically separate sites (s.c. and i.p.). Drugs are administered i.p. (mice are treated daily for up to 4 days), fibers are removed 2 days later (total time in vivo is 6 days), and chemosensitivity is assessed using a modified MTT assay in vitro (8). Compounds are submitted for more extensive in vivo evaluation in xenografts if they demonstrate activity at both the s.c. and i.p. sites. Responses at the i.p. site might be expected to reflect in vitro chemosensitivity (although they are unlikely to be identical as drug exposure parameters will be different due to drug clearance from the peritoneal cavity), as it is unlikely that drug delivery problems would occur after an i.p. drug administration. Responses at the s.c. site after i.p. drug administration, however, may indicate that the new agent possesses both good bioavailability and pharmacological properties. Drug delivery to tumors is dependent on several factors, one of which is the presence of an established blood supply. Blood vessel development to hollow fibers implanted s.c. has not been reported previously, raising concerns about the efficiency of drug delivery and the subsequent effects this may have on chemosensitivity, bearing in mind the role that drug C × T values play in determining chemosensitivity (9). Lack of activity at the s.c. site may, therefore, be caused by poor drug delivery as a result of lack of blood vessel development; if this is the case, the validity of the test system is called into question. Tumor cells in hollow fibers should theoretically be able to induce an angiogenic response as the molecular weight cutoff point of the fiber walls is large enough (M, 500,000) to allow for appropriate growth factors to pass through the fiber wall. Present protocols for using the hollow fiber assay involve both short-term in vitro (1 day) and in vivo (6 day) cultivation, which may be too short to enable angiogenesis to occur. The aims of this study, therefore, were: (a) to determine whether hollow fibers implanted s.c. can develop a blood supply if postimplantation times are extended beyond the 6-day period presently used; and (b) to determine what effect the presence or absence of a vascular network has on both drug delivery and chemosensitivity in a murine colon tumor model.

Materials and Methods

Materials. Poly-Vinyl-Idene-Fluoride Spectra/Por Hollow fibers (internal diameter = 1 mm with a molecular weight cutoff point of M, 500,000) were purchased from Spectrum Medical, Inc. (Houston, TX). Doxorubicin, MTT, and DMSO were purchased from Sigma (Poole, Dorset, United Kingdom). Solvents for high-performance liquid chromatography analysis of doxorubicin were of analytical grade and were purchased from Fisher Scientific (Loughborough, United Kingdom). All cell culture material was purchased from Life Technologies, Inc. (Paisley, United Kingdom).

Animals. Pure strain NMRI mice (6–8 weeks of age) were obtained from B & K Universal, Ltd. (Hull, United Kingdom) and kept under 12-hour cycles of light and darkness with access to a CRM pellet diet (Special Diet Services, Witham, United Kingdom) and water ad libitum. All animal experiments were conducted under appropriate animal licenses issued by the Home Office (London, United Kingdom), and United Kingdom Coordinating Committee on Cancer Research guidelines were followed throughout (10).

Cell Culture. MAC 15A cells were obtained from an ascitic murine adenocarcinoma of the colon, as described elsewhere (9), and routinely maintained as monolayer cultures in RPMI 1640 supplemented with FCS (10%), sodium pyruvate (2 mM), Penicillin/Streptomycin (50 IU ml−1/50 µg ml−1), and L-glutamine (2 mM) and buffered with HEPES (25 mM). Fibers were transferred to a T-25 flask containing growth medium and incubated at 37°C for a period of 14 days under constant agitation (80 rpm) on an orbital shaker, with daily changes of medium. Histological analysis of fibers after 14 days of culture in vitro demonstrated that fibers contain a central necrotic core (data not shown).
Fig. 1. Morphological appearance of blood vessel development to hollow fibers implanted s.c. into NMRI mice. A, typical appearance of subdermal vasculature in the absence of fibers. Blood vessel development to fibers containing MAC 15A cells on days 4 (B), 7 (C), 14 (D), 21 (E), 28 (F), and 32 (G) after implantation at the s.c. site. H, absence of blood vessel development to fibers containing medium only (28 days after implantation s.c.).
Blood Vessel Development to Hollow Fibers. As the production of growth factors such as vascular endothelial growth factor is enhanced under hypoxic conditions (11), implantation of "mature" fibers containing central necrotic cores (7) may further stimulate angiogenesis. After 14 days of culture in vitro, hollow fibers (containing central necrotic cores) were implanted s.c. using a sterile trocar (3 fibers/mouse at the same site). At various times after implantation, mice were sacrificed by cervical dislocation, a central midline incision made, and the skin peeled back to reveal the fibers in situ. Blood vessel development to fibers was recorded photographically. Fibers containing medium alone were also implanted s.c. to serve as controls.

Chemosensitivity Studies. Fibers containing MAC 15A cells were implanted s.c. and i.p. using a sterile trocar (3 fibers/site). For implantation of fibers i.p., mice were anesthetized before implantation using a trocar. Doxorubicin was administered to mice (10 mg kg−1 i.p.) on days 4 or 28 after fiber implantation at both the l.p. and s.c. sites. Fibers were retrieved from mice 6 days after drug administration (time from first treatment to fiber removal is comparable with protocols used by the NCI), and chemosensitivity assessed using a modified MTT assay, details of which are described elsewhere (8). Chemosensitivity was expressed in terms of the percentage of cell survival taking the absorbance of control fibers (mice treated with drug vehicle only) to represent 100% cell survival. Statistical comparisons were made using Student’s t test.

Drug Analysis. Mice bearing fibers containing MAC 15A cells implanted s.c. and i.p. were treated with doxorubicin (10 mg kg−1, i.p.) either on days 4 or 28 after implantation. Fibers were removed 1 h after drug administration, the heat seals removed, and the contents expelled by air into a preweighed Eppendorf tube. Fibers were flushed with PBS (50 μl) into the same tube, and protein was precipitated by the addition of 100 μl MeCN:methanol (3:1). After centrifugation at 7,000 × g for 5 min, doxorubicin concentration (expressed as amount/weight of fiber contents) was determined by high-performance liquid chromatography, as described elsewhere (12). Control fibers (containing medium only) were treated in an identical manner to fibers containing MAC 15A cells, and results were expressed in terms of amount/weight of fiber contents. Statistical comparisons were made using Student’s t test.

Results and Discussion

The hollow fiber assay presently in use by the NCI is a relatively short-term assay conducted over a period of 6 days in vivo. During this time period, the development of a vascular supply to hollow fibers has not been reported, and Hollingshead et al. (8) conclude that the hollow fiber assay is not appropriate for studying host/tumor interactions such as angiogenesis. By extending the postimplantation time in vivo, however, extensive vascular networks to hollow fibers containing MAC 15A cells do develop over a period of 28–32 days (Fig. 1). Similar results were obtained with other MAC cell lines (MAC 13 in NMRI mice and MCF-7 in NCR nude mice) with extensive vascular networks established 28 days after implantation (data not shown). The development of a blood supply is dependent on the presence of tumor cells, as control fibers containing medium only do not stimulate angiogenesis over a similar time scale (Fig. 1). Although the original aim of this study was not to develop and fully characterize a novel model of angiogenesis, the results of this study suggest that the hollow fiber techniques could be used in the field of angiogenesis and the search for angiogenesis inhibitors. It has certain theoretical advantages over presently available techniques, in that genetically well-characterized cells (including patterns of growth factor expression) can be implanted into fibers, and that these cells can be retrieved intact at various stages after implantation without host cell infiltration. Additional studies are required, however, to determine whether angiogenesis in the hollow fiber model has advantages over presently existing techniques (13), particularly with respect to quantitation, the use of windows to observe blood vessel development, and the use of fibers containing gels impregnated with growth factors.

The presence or absence of a vascular supply to hollow fibers implanted s.c. has a significant effect on both drug delivery (Table 1) and chemosensitivity (Table 2). In terms of drug delivery, the concentration of doxorubicin in hollow fibers (s.c.) containing MAC 15A cells (Table 1) is significantly higher when doxorubicin is administered 28 days after implantation (0.43 ± 0.35 ng mg−1) compared with the same fibers treated 4 days after implantation (<0.015 ng mg−1). In hollow fibers (s.c.) containing medium only, no significant differences exist in doxorubicin levels inside fibers treated either 28 or 4 days after implantation (Table 1), which reflects the fact that development of a blood vessel supply does not occur in blank fibers (Fig. 1). Similarly, in fibers implanted i.p. post implantation, times have no significant effect on drug concentrations inside the fiber, although much greater concentrations are achieved compared with fibers implanted s.c. (Table 1). The relationship between drug distribution and blood vessel development is also reflected in terms of chemosensitivity in that s.c. fibers treated when a vascular supply is established (day 28) are more sensitive to doxorubicin than s.c. fibers treated in the absence of a vascular supply (Table 2). No significant difference exists between the response of MAC 15A cells in fibers implanted i.p. for 4 or 28 days before drug administration (Table 2).

The results of this study demonstrate that the treatment of hollow fibers s.c. with doxorubicin in the absence of an established blood supply gives rise to a false negative result due largely to the fact that drug delivery is impaired. In terms of drug screening, experimental models must meet several criteria, one of which being that the predictive value of the model is such that false negative results are kept to a minimum. In the case of the hollow fiber assay, no published information is available on the predictive value of the hollow fiber assay in terms of whether or not responses in hollow fibers correlate with activity in xenografts. Hollingshead et al. (8) have demonstrated activity, however, against fibers implanted s.c. for several standard anticancer agents, although it is not known how drugs reach this site in the absence of a vascular supply. As impaired drug delivery to tumors is a major obstacle for an effective anticancer drug therapy, it could be argued that compounds that are active against hollow fibers at the s.c. site have the highly desirable property of being efficiently delivered to tumors in the absence of a good blood supply. In addition, modifications to the hollow fiber assay to commence chemotherapy at various stages after implantation would lead to more compounds going through to testing against xenograft models, resulting in possible logistical problems for large-scale screening programs. Nevertheless, the results of this study raise the possibility that potentially useful compounds may be missed and suggest that additional studies to address the issues of false negative prediction rates and the feasibility of screening potentially more compounds against xenografts are required.

<table>
<thead>
<tr>
<th>Site of implantation</th>
<th>% Cell survival (treatment time, day 4)</th>
<th>% Cell survival (treatment time, day 28)</th>
</tr>
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<tbody>
<tr>
<td>s.c.</td>
<td>97.6 ± 20.1</td>
<td>47.8 ± 14.3</td>
</tr>
<tr>
<td>i.p.</td>
<td>10.1 ± 6.5</td>
<td>2.8 ± 2.0</td>
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* Each value represents the mean ± SD of at least three independent experiments.
In conclusion, this study has demonstrated that hollow fibers containing tumor cells can induce angiogenesis in mice and this has direct implications for drug delivery and chemosensitivity. These results suggest that the treatment of fibers before blood vessel development occurs may be a source of false negative predictions, and studies to address this question need to be conducted. Finally, the demonstration that hollow fiber tumor models can induce an angiogenic response in the host suggests that this model may have some use in the search for antiangiogenic drugs, although considerable refinement of the assay to address questions such as quantitation of blood vessel development need to be addressed.

References


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