Intratumour heterogeneity in the uptake of macromolecular therapeutic agents in human melanoma xenografts

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Intratumour heterogeneity in the uptake of blood-borne technetium-labelled human serum albumin (\(\text{\textsuperscript{99m}}\text{Tc-HSA}\)) was studied in human melanoma xenografts in an attempt to identify transport barriers leading to inadequate and heterogeneous uptake of macromolecular therapeutic agents in tumours. The Bioscope imaging system, which can detect the distribution of \(\text{\textsuperscript{99m}}\text{Tc}\) in \(10\mu\text{m}\)-thick tissue sections with a spatial resolution of just above \(50\mu\text{m}\), was used to image the \(\text{\textsuperscript{99m}}\text{Tc-HSA}\) uptake. Xenografted tumours of four human melanoma cell lines were included in the study. Significant intratumour heterogeneity in the uptake of \(\text{\textsuperscript{99m}}\text{Tc-HSA}\) was detected. The heterogeneity had two distinctly different components, one random and one radial component. The uptake was lowest in the centre of the tumours and increased towards the tumour periphery. This radial heterogeneity was superimposed by a random heterogeneity, that is, spots with high uptake colocalised with spots with high vascular density and regions without significant uptake colocalised with necrotic regions. The magnitude of the heterogeneity did not change significantly with time after the administration of \(\text{\textsuperscript{99m}}\text{Tc-HSA}\). The tumours showed a random and a radial heterogeneity in blood perfusion similar to that in the uptake of \(\text{\textsuperscript{99m}}\text{Tc-HSA}\). The observations reported here suggest that the intratumour heterogeneity in the distribution of \(\text{\textsuperscript{99m}}\text{Tc-HSA}\) was initiated primarily because of heterogeneity in the supply of \(\text{\textsuperscript{99m}}\text{Tc-HSA}\) through the microvasculature, and that the presence of severe transport barriers in the tumour interstitium prevented significant equalisation of the initial heterogeneity with time. Consequently, strategies for improving the delivery of macromolecular therapeutic agents to tumours should focus on increasing the tumour blood perfusion to increase the total uptake and improving the diffusion conditions in the tumour interstitium to diminish the heterogeneity in the uptake.

Keywords: macromolecule uptake; melanoma; microvasculature; interstitium; blood supply; perfusion; transport mechanisms

Novel strategies for the treatment of cancer involving macromolecular therapeutic agents such as growth factors, monoclonal antibodies, immunomodulators, oligonucleotides, gene therapy vectors and encapsulated drugs have been developed recently (Jain, 1998). Preclinical studies of the therapeutic usefulness of such agents in several \textit{in vitro} and \textit{in vivo} cancer models have given promising results (Yuan, 1998). However, inadequate and heterogeneous uptake in tumour tissue has been shown to be a major obstacle for efficient use of macromolecules in clinical cancer therapy (Yuan, 1998; Jain, 2001). Improved strategies are therefore needed to increase the macromolecule delivery to tumours. The uptake of macromolecular therapeutic agents in tumour tissue depends on the physical and chemical properties of the macromolecule and the biology of the tissue. Macromolecular properties exerting a significant influence on the uptake include molecular weight, shape, charge and affinity for binding to stromal and parenchymal tissues (Dellian \textit{et al}., 2000). Biological factors that may prevent adequate uptake include low microvascular density, poor perfusion, low transvascular permeability, high interstitial fluid pressure and a dense, well-organised extracellular matrix (Hobbs \textit{et al}., 1998). However, the mechanisms regulating the uptake are not well understood. Novel strategies for increasing the delivery of therapeutic agents can probably not be developed without identification of the barriers limiting the uptake. Such identification requires careful studies with well-characterised experimental tumours and macromolecules with well-known physical and chemical properties.

The mechanisms governing the uptake of macromolecular therapeutic agents in human melanoma xenografts are currently being investigated in our laboratory, using albumin as a model molecule for macromolecular therapeutic agents (Graff \textit{et al}., 2000, 2001; Bjørnæs and Rofstad, 2001). We have previously measured the global uptake of blood-borne albumin, and found that it was strongly correlated to the extracellular volume fraction of the tumour tissue (Graff \textit{et al}., 2000) and was not limited by the permeability of the microvascular wall (Bjørnæs and Rofstad, 2001; Graff \textit{et al}., 2001). In the present work, the intratumour heterogeneity in the uptake of technetium-labelled human serum albumin (\(\text{\textsuperscript{99m}}\text{Tc-HSA}\)) was studied and related to the intratumour heterogeneity in necrosis, vascular density, blood perfusion and extracellular volume fraction. Human melanoma xenografts having retained essential biological features of the donor patients’ tumours, including histology, growth rate and vascularisation, have been established and characterised in our laboratory (Rofstad, 1994).
Heterogeneity in macromolecule uptake

MATERIALS AND METHODS

Mice and tumours

Adult (8–12 weeks of age) female BALB/c-nu/nu mice, bred at our research institute, were used as host animals for xenografted tumours. The mice were maintained under specific pathogen-free conditions at constant temperature (24–26 °C) and humidity (30–50%). Sterilised food and tap water were given ad libitum.

Four human melanoma lines (A-07, D-12, R-18 and U-25), described in detail elsewhere (Rofstad, 1994), were included in the study. Tumours were initiated from exponentially growing monolayer cultures verified to be free from Mycoplasma contamination. The cells were cultured in RPMI-1640 medium (25 mM HEPES and l-glutamine) supplemented with 13% bovine calf serum, 250 mg l⁻¹ penicillin and 50 mg l⁻¹ streptomycin. Approximately 3.5 × 10⁶ cells in 10 μl of Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution were inoculated intradermally into the flanks of mice by using a 100-μl Hamilton syringe. The tumours were deposited above the subcutaneous muscle tissue in the deeper part of the dermis. The growth and histological appearance of the tumours have been described elsewhere (Rofstad, 1994). D-12 and U-25 tumours develop necrotic regions during growth, whereas A-07 and R-18 tumours do not. Tumours with volumes within the range of 300–600 mm³ were subjected to investigation. Tumour volume (V) was calculated as \( V = \pi ab^2 \), where \( a \) is the longer and \( b \) is the shorter of two perpendicular diameters, measured with callipers.

Animal experiments were approved by the Institutional Committee on Research Animal Care and were performed according to the ethical standards of the UKCCCR ‘Guidelines for the Welfare of Animals in Experimental Neoplasia’ (Workman et al, 1998).

Bioscope

The Bioscope system (IDE AS, Oslo, Norway), designed to image the distribution of isotopes in tissue sections with high spatial resolution (Overdick et al, 1997), was used to study intratumour heterogeneity in macromolecule uptake, extracellular volume fraction and blood perfusion (Figure 1). The sensor of this system is a 300-μm-thick double-sided silicon strip detector with a sensitive area of 32 × 32 mm. The detector has 640 strips in each direction, that is, a strip pitch of 50 μm. Four tumour sections can be imaged simultaneously, as illustrated in Figure 1A. The positions of ionising events are registered by the detector and transferred to a computer. Low-energy β-emitters, such as ⁹⁹mTc, give images with a spatial resolution of just above 50 μm. Quantitative analysis of images was performed by using the analySIS image processing system (Soft Imaging System GmbH, Münster, Germany). The radial distribution of activity was determined by dividing the tumour images into five sectors and analysing each sector separately, as illustrated in Figure 1B. The sectors were bounded by lines drawn at distances of \( nR/5 \) from the tumour centre, where \( R \) is the tumour radius and \( n \) is the sector number. The skin surrounding the tumours was excluded from the analyses.

Macromolecule uptake

⁹⁹mTc-HSA was diluted in 0.9% saline and administered intravenously to tumour-bearing mice in a bolus dose of 32 mg kg⁻¹ (approximately 8 × 10⁶ MBq kg⁻¹). The mice were killed by cervical dislocation 1, 5, 10, 30, 60, 120 or 180 min after the administration, and the tumours were frozen in liquid nitrogen and cut into two halves. One of the halves was subjected to measurement of activity in a gamma counter (Wallac Oy, Turku, Finland) to ascertain that the kinetics of the global uptake was consistent with previous measurements. Frozen sections of 10-μm thickness were prepared from the other halves, using standard procedures. The spatial distribution of activity in these sections, that is, the concentration of ⁹⁹mTc-HSA, was imaged with the Bioscope system and used as a parameter for the intratumour heterogeneity in macromolecule uptake. The sections were stained with haematoxylin and eosin after the imaging and examined by light microscopy.

Extracellular volume fraction

⁹⁹mTc-HSA in doses similar to those described above was administered intravenously to tumour-bearing mice with ligated renal arteries. The procedure used for the ligation of renal arteries has been described elsewhere (Bjørnæs et al, 2000). The mice were killed and the tumours were frozen in liquid nitrogen 3 h after the administration.
administration of 99mTc-HSA. Frozen sections (10-μm thick) were prepared from the tumours, and the spatial distribution of activity in the sections, that is, the concentration of 99mTc-HSA, was imaged with the Bioscope system and used as a parameter for the intratumour heterogeneity in extracellular volume fraction.

Blood perfusion

Na99mTcO4 was administered intravenously to tumour-bearing mice in a bolus dose (approximately $8 \times 10^2$ MBq kg\(^{-1}\)). The mice were killed and the tumours were frozen in liquid nitrogen 45 s after the administration of Na99mTcO4. Frozen sections (10-μm thick) were prepared from the tumours, and the spatial distribution of activity in the sections, that is, the concentration of Na99mTcO4, was imaged with the Bioscope system and used as a parameter for the intratumour heterogeneity in perfusion.

Statistical analysis

Results are presented as arithmetic mean±s.e.m. The Pearson Product Moment Correlation test was used under conditions of normality and equal variance to determine the strength of correlations between variables. The statistical analysis was performed by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

Qualitative studies

Qualitative examinations of Bioscope images revealed significant intratumour heterogeneity in the uptake of 99mTc-HSA. The heterogeneity had two distinctly different components, one random and one nonrandom radial component. The uptake was lowest in the centre of the tumours and increased towards the tumour periphery. This radial heterogeneity was superimposed by a random heterogeneity. A-07 and R-18 tumours showed spots with high uptake and randomly distributed within the tissue. D-12 and U-25 tumours showed randomly distributed regions without significant uptake in addition to spots with high uptake.

Bioscope images of 99mTc-HSA activity were superimposed on histological images in an attempt to identify the causes of the random component of the heterogeneity (Figure 2). Spots with high uptake colocalised with spots with high vascular density, either the tumour host was killed minutes (Figure 2A) or hours (Figure 2B) after the administration of 99mTc-HSA. Regions without significant uptake colocalised with necrotic regions independent of the time between the administration of 99mTc-HSA and the killing of the tumour host (Figure 2C).

Moreover, qualitative examinations of Bioscope images of Na99mTcO4 activity revealed an intratumour heterogeneity in blood perfusion similar to that in the uptake of 99mTc-HSA. The perfusion was lowest in the centre of the tumours and increased towards the tumour periphery. Spots with high perfusion colocalised with spots with high vascular density and regions without perfusion co-localised with necrotic regions (data not shown).

Quantitative studies

The Bioscope images were divided into five sectors (Figure 1B) to quantify the radial heterogeneity in the uptake of 99mTc-HSA. Representative data for A-07 tumours frozen 1, 30 or 180 min after the administration of 99mTc-HSA are presented in Figure 3, illustrating three general observations: (a) the concentration of 99mTc-HSA increased with time, (b) the concentration of 99mTc-HSA increased linearly with the distance from the tumour centre.
and (c) the ratio of the concentration of $^{99m}$Tc-HSA in the tumour periphery and the tumour centre was independent of time within the time interval of $1-180$ min. Similar analysis revealed that these three observations were also valid for R-18 tumours (data not shown). Consequently, the magnitude of the radial heterogeneity in the uptake of $^{99m}$Tc-HSA was independent of time within the time interval of $1-180$ min in both A-07 and R-18 tumours, that is, tumours without necrotic regions. Moreover, the uptake of $^{99m}$Tc-HSA and the magnitude of the radial heterogeneity in the uptake were independent of tumour volume within the volume range studied here, that is, $300-600$ mm$^3$, in both A-07 and R-18 tumours (data not shown).

Data for individual tumours were normalised and pooled to compare the radial heterogeneity in the uptake of $^{99m}$Tc-HSA in A-07 and R-18 tumours (Figure 4). A significant difference between the two tumour types could not be detected; the concentration of $^{99m}$Tc-HSA in the tumour periphery was higher than that in the tumour centre by a factor of approximately 1.5 in both tumour lines ($P < 0.05$). The same procedure was used to quantify the radial heterogeneity in the uptake of $^{99m}$Tc-HSA in D-12 and U-25 tumours, that is, tumours showing significant necrotic regions (Figure 5). The quantitative analysis did not reveal a significant radial gradient in the uptake of $^{99m}$Tc-HSA in these tumour lines ($P > 0.05$), although the qualitative examination showed that the uptake in non-necrotic tissue was substantially higher in the tumour periphery than in the tumour centre. Consequently, the data in Figure 5 most likely reflect that necrotic regions without
significant uptake of $^{99m}$Tc-HSA had a random radial distribution and were sufficiently large to govern the overall radial heterogeneity in the uptake of $^{99m}$Tc-HSA.

The radial distributions in blood perfusion (Figure 6) and extracellular volume fraction (Figure 7) were quantified in A-07 and R-18 tumours in an attempt to identify causes of the radial heterogeneity in the uptake of $^{99m}$Tc-HSA. The perfusion was lowest in the centre of the tumours and increased towards the tumour periphery. A-07 tumours showed approximately three-fold higher perfusion in the periphery than in the centre ($P = 0.05$), whereas in R-18 tumours, the perfusion in the periphery was higher than that in the centre by a factor of approximately 1.5 ($P = 0.05$). The radial heterogeneity in extracellular volume fraction, on the other hand, was weak in both tumour lines. The extracellular volume fraction decreased slightly towards the tumour periphery in A-07 tumours ($P = 0.030$) and increased slightly with the distance from the tumour centre in R-18 tumours ($P = 0.035$).

**DISCUSSION**

Intratumour heterogeneity in the uptake of $^{99m}$Tc-HSA was studied in human melanoma xenografts by utilising the unique properties of the newly developed Bioscope imaging system (Overdick et al., 1997). This system made it feasible to image the activity of $^{99m}$Tc-HSA in 10-μm-thick tumour sections at a spatial resolution of just above 50 μm. The images could be subjected to quantitative analysis to determine the uptake of $^{99m}$Tc-HSA in any freely selected tumour subregion. Moreover, correlations between the uptake of $^{99m}$Tc-HSA and histological characteristics could be searched for by superimposing Bioscope images on histological images obtained from the same tumour sections.

Studies of intratumour heterogeneity in the uptake of macromolecules using a radiolabelled molecule as a probe require specific precautions to avoid experimental artefacts and incorrect interpretation of data. Degradation of the probe, resulting in unbound and hence freely diffusible activity, may generate data indicating that the heterogeneity is lower than the true heterogeneity. $^{99m}$Tc-HSA was used as a model molecule for macromolecular therapeutic agents in the present work. Intratumour heterogeneity in $^{99m}$Tc-HSA concentration was seen within a few minutes after the administration of the probe, and this initial heterogeneity was maintained at unchanged magnitude throughout the whole observation period of 180 min. In contrast, control experiments with Na$^{99m}$TcO$_4$ showed that the initial heterogeneity in Na$^{99m}$TcO$_4$ concentration decayed rapidly with time, resulting in a homogeneous distribution within less than an hour. Consequently, degradation of $^{99m}$Tc-HSA probably did not represent a serious problem in our study.

Significant intratumour heterogeneity in the uptake of $^{99m}$Tc-HSA was detected. The heterogeneity had two distinctly different components, one random and one radial component. In
non-necrotic tissue, the uptake was lowest in the centre of the
tumours and increased towards the tumour periphery. The radial
distribution of $^{99m}$Tc-HSA was initiated within minutes after the
administration of $^{99m}$Tc-HSA and was maintained at least until
maximum uptake was attained, approximately 180 min later.
Mathematical modelling has suggested that radial heterogeneity
in the uptake of macromolecules in tumours could be because of
radial heterogeneity in blood perfusion, histopathological char-
acteristics, microvascular permeability and/or interstitial fluid
pressure (Baxter and Jain, 1988, 1990; Jain and Baxter, 1988;
Fujimori et al, 1989; Thomas et al, 1989).
The radial intratumour heterogeneity in blood perfusion and
extracellular volume fraction was determined experimentally in the
present work by using $^{99m}$Tc-labelled molecules as tracers and the
Bioscope imaging system for detection. Experimental studies with
the same melanoma lines of intratumour heterogeneity in the
permeability of the microvasculature to Gd-labelled HSA and
intratumour heterogeneity in interstitial fluid pressure have been
reported previously (Bjørnæs and Rofstad, 2001; Rofstad et al,
2002). As discussed below, the biological factors leading to the
radial heterogeneity in the uptake of $^{99m}$Tc-HSA can be identified
from these studies.

The radial heterogeneity in the uptake of $^{99m}$Tc-HSA appeared
within minutes after the administration of $^{99m}$Tc-HSA in both A-07
and R-18 tumours. The blood perfusion in these tumours showed a
radial gradient similar to that in the uptake of $^{99m}$Tc-HSA. These
two observations together strongly suggest that intratumour
heterogeneity in the supply of $^{99m}$Tc-HSA through the micro-
vasculature contributed significantly to the initiation of the radial
gradients in $^{99m}$Tc-HSA uptake.

We have reported previously that our melanoma lines show an
intratumour heterogeneity in the uptake of albumin that is
governed primarily by intertumour differences in extracellular
volume fraction (Graff et al, 2000). However, intratumour
heterogeneity in extracellular volume fraction probably did not
contribute significantly to the radial gradients in $^{99m}$Tc-HSA
uptake, since neither A-07 nor R-18 tumours showed strong radial
gradients in extracellular volume fraction.

Quantitative studies of the permeability of the microvascular
wall of A-07 and R-18 tumours to Gd-labelled HSA have been
reported elsewhere (Bjørnæs and Rofstad, 2001). The effective
microvascular permeability constant was found to be extremely
high, suggesting that the uptake of HSA in these tumours is
not inhibited significantly by the microvascular wall. However,
intratumour heterogeneity in the effective microvascular
permeability constant was detected, but this heterogeneity
was random. Consequently, intratumour heterogeneity in the
permeability of the microvascular wall probably did not con-
tribute significantly to the radial gradients in $^{99m}$Tc-HSA uptake
measured here.

Measurements of the radial heterogeneity in interstitial fluid
pressure (IFP) in A-07 tumours have been reported recently
(Rofstad et al, 2002). These measurements showed that the IFP
increased with increasing distance into the tumours until a plateau
was reached at a depth of 0.75 mm, that is, the tumours showed a
steep IFP gradient in the periphery and relatively uniform IFP
values at depths beyond 0.75 mm. A depth of 0.75 mm corresponds
to a normalised radius $>0.8$ in the tumours used here, implying
that the radial gradients in $^{99m}$Tc-HSA uptake did not correspond
to the radial gradients in IFP (Rofstad et al, 2002). One possible
explanation is that convection may not be the most important
transport mechanism for $^{99m}$Tc-HSA (Perl, 1975). Another possible
explanation is that $^{99m}$Tc-HSA delivered in the tumour periphery
was transported out of the tissue and into the surrounding skin
because of the steep IFP gradient in the tumour periphery. In any
case, the radial gradients in $^{99m}$Tc-HSA uptake seen here were not
primarily a consequence of radial gradients in IFP.

The radial gradients in the uptake of $^{99m}$Tc-HSA seen shortly
after the administration of $^{99m}$Tc-HSA were maintained for at least
180 min, that is, until maximum global uptake was reached. This
observation shows that the interstitial transport of $^{99m}$Tc-HSA
from peripheral tumour regions with high blood perfusion to
central tumour regions with low blood perfusion was inefficient. A
dense and well-organised extracellular matrix may have prevented
efficient transport by diffusion (Hobbs et al, 1998; Krol et al, 1999),
whereas convection may have caused transport from the centre to
the periphery of the tumours, that is, in the opposite direction
(Jain and Baxter, 1988).

Consequently, the studies discussed here collectively suggest
that the tumour microvasculature and the tumour interstitium
represented significant transport barriers for $^{99m}$Tc-HSA in our
melanoma xenografts, causing radial gradients in the uptake of
$^{99m}$Tc-HSA in non-necrotic tissue. The gradients were initiated
primarily because of radial heterogeneity in the supply of $^{99m}$Tc-
HSA through the microvasculature. This radial heterogeneity was
maintained at least until maximum global uptake was attained,
mainly because of the presence of significant transport barriers in
the tumour interstitium preventing equalisation of the initial
heterogeneity with time.

The random component of the intratumour heterogeneity in the
uptake of $^{99m}$Tc-HSA was most likely also caused by the
same transport barriers, that is, heterogeneous supply through
the tumour microvasculature and inadequate transport through
the tumour interstitium. This conclusion follows from the
observations that spots with high $^{99m}$Tc-HSA uptake colocalised
with spots with high vascular density and regions without
significant $^{99m}$Tc-HSA uptake colocalised with necrotic regions,
that is, regions at long distances from functional vessels.

If our melanoma xenografts are representative models of cancer
in humans, the study reported here suggests that strategies for
improving the delivery of macromolecular therapeutic agents to
tumours should focus on increasing the tumour blood perfusion
and improving the diffusion conditions in the tumour interstitium.
The increased perfusion may lead to an enhancement of the total
macromolecule uptake and improved diffusion conditions may
lead to a more homogeneous macromolecule distribution in the
tumour tissue.

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