Imaging the tumor microenvironment

THE DYNAMICS AND MODIFICATION OF HYPOXIA

Anna Ljungkvist
Imaging the tumor microenvironment:

The dynamics and modification of hypoxia

Anna Ljungkvist

Umeå 2003
Cover: Milstolpe, milstone, mijlpaal, at road 1078, Nymölla, Sweden.
Built 1780, when Gustav III was king and Gabriel Sparre was county governor.
At those days 1 Swedish “mil” was 36 000 feet e.g. about 10688 m.

© Anna Ljungkvist
Printed in Sweden by Print & Media, Umeå universitet
Umeå 2003:302018
ISBN 91-7305-432-1
ISSN 0346-6612
To my

Mother, Father and Brother

“In science one tries to tell people, in such a way as to be understood by everyone, something that no one ever knew before. But in poetry, it's the exact opposite.”

Paul Dirac
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>7</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF PUBLICATIONS</td>
<td>10</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>11</td>
</tr>
<tr>
<td>General background</td>
<td>11</td>
</tr>
<tr>
<td>Tumor microenvironment</td>
<td>12</td>
</tr>
<tr>
<td>Tumor vasculature</td>
<td>13</td>
</tr>
<tr>
<td>Organization</td>
<td>13</td>
</tr>
<tr>
<td>Prognostic value</td>
<td>14</td>
</tr>
<tr>
<td>Vascular targeting</td>
<td>14</td>
</tr>
<tr>
<td>Perfusion</td>
<td>15</td>
</tr>
<tr>
<td>Proliferation</td>
<td>16</td>
</tr>
<tr>
<td>Proliferation patterns and prognosis</td>
<td>17</td>
</tr>
<tr>
<td>Tumor hypoxia</td>
<td>18</td>
</tr>
<tr>
<td>Definitions of hypoxia</td>
<td>18</td>
</tr>
<tr>
<td>Radiobiological hypoxia</td>
<td>19</td>
</tr>
<tr>
<td>Hypoxia and prognosis</td>
<td>20</td>
</tr>
<tr>
<td>Assays for detection of hypoxia</td>
<td>21</td>
</tr>
<tr>
<td>Patho-physiology of hypoxia</td>
<td>22</td>
</tr>
<tr>
<td>Dynamics of hypoxic cells</td>
<td>24</td>
</tr>
<tr>
<td>Hypoxia and malignant progression</td>
<td>25</td>
</tr>
<tr>
<td>Cell loss, apoptosis and necrosis</td>
<td>27</td>
</tr>
<tr>
<td>Relation between proliferation, hypoxia and cell loss</td>
<td>27</td>
</tr>
<tr>
<td>AIMS OF THE PRESENT STUDY</td>
<td>29</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>31</td>
</tr>
<tr>
<td>Tumor models</td>
<td>31</td>
</tr>
<tr>
<td>Exogenous cell markers and administered drugs</td>
<td>31</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>34</td>
</tr>
<tr>
<td>Image acquisition</td>
<td>36</td>
</tr>
<tr>
<td>Resolution</td>
<td>36</td>
</tr>
</tbody>
</table>
**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanning and thresholding, 8-bit system</td>
<td>38</td>
</tr>
<tr>
<td>Scanning and thresholding, 12-bit system</td>
<td>38</td>
</tr>
<tr>
<td>Post processing of images exported from IP-Lab to TCL-image</td>
<td>40</td>
</tr>
<tr>
<td>Quantitative parameters:</td>
<td>41</td>
</tr>
<tr>
<td><strong>SUMMARY OF RESULTS</strong></td>
<td>45</td>
</tr>
<tr>
<td>Characterization of the tumor microenvironment (paper I)</td>
<td>45</td>
</tr>
<tr>
<td>The lifespan of hypoxic cells in three different tumor lines (paper II)</td>
<td>46</td>
</tr>
<tr>
<td>Dynamic measurements of hypoxia after oxygenation modifying treatment (paper III)</td>
<td>46</td>
</tr>
<tr>
<td>Dynamic changes in hypoxia after treatment with the hypoxic cytotoxin hydralazine (IV)</td>
<td>47</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>49</td>
</tr>
<tr>
<td>Tumor microenvironment and model systems</td>
<td>49</td>
</tr>
<tr>
<td>Double hypoxic marker assay</td>
<td>50</td>
</tr>
<tr>
<td>Binding profiles of the two hypoxic markers</td>
<td>50</td>
</tr>
<tr>
<td>Spatial match/mismatch of the hypoxic markers</td>
<td>51</td>
</tr>
<tr>
<td>Detection of perfusion limited (acute) hypoxia</td>
<td>52</td>
</tr>
<tr>
<td>Hypoxic patterns</td>
<td>53</td>
</tr>
<tr>
<td>Hypoxic lifespan and its relation to hypoxic patterns</td>
<td>53</td>
</tr>
<tr>
<td>Hypoxia and anti-cancer treatments</td>
<td>54</td>
</tr>
<tr>
<td>Reproducibility of results: impact of different image analysis systems and staining protocols</td>
<td>55</td>
</tr>
<tr>
<td>Future Aims</td>
<td>56</td>
</tr>
<tr>
<td><strong>CONCLUSIONS</strong></td>
<td>59</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>61</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>63</td>
</tr>
</tbody>
</table>
ABSTRACT

The tumor vasculature is poor and heterogeneous which may result in inadequate oxygenation and changed energy status. In addition the balance between cell proliferation and the rate of cell death is disturbed, which results in tumor growth. The aims of this study were 1) to gain more insight into the relation between tumor vascularity, hypoxia, and proliferation in solid tumors, and 2) to study the changes and dynamics of tumor oxygenation in relation to the vascular architecture within individual tumors. For this purpose a double hypoxic marker method was developed, which was subsequently used to 3) determine the turnover rate of hypoxic cells in three different tumor models and 4) to study the effect of cytotoxic drugs on tumor hypoxia and cell death.

Solid tumor models grown in mice were used. The tumor microenvironment was investigated with exogenous cell markers for hypoxia (pimonidazole and CCI-103F), cell proliferation (BrdUrd) and blood perfusion (Hoechst 33342). The vasculature and the exogenous cell markers were visualized with immunohistochemical techniques. The tumor sections were scanned and quantified with an image analysis system consisting of a fluorescence microscope, CCD camera and image analysis software.

The spatial organization of hypoxia, proliferation, and tumor vasculature was analyzed in several xenograft lines. The study revealed two main hypoxic patterns that seemed to be the consequence of complex relations between vasculature, oxygen delivery, proliferation, and cell loss. The novel double hypoxic cell marker method, with sequential injection of two hypoxic markers, was developed to study dynamic changes of the tumor oxygenation. Based on varying injection intervals between the markers the hypoxic cell half-life was determined in three tumor lines, and ranged from 17 to 49 hours. Intra-tumoral changes in oxygenation status upon oxygen modifying treatments were measured with the double hypoxic marker method. Both decreased levels of tumor hypoxia after carbogen breathing (95%O2 and 5% CO2) and increased levels of tumor hypoxia, as a result of reduced tumor perfusion after hydralazine treatment was detected. Finally the double hypoxic marker assay was used to analyze the effects of the hypoxic cytotoxin tirapazamine in relation to the hypoxic cell population, which caused a reversible decrease of the hypoxic fraction.

The results presented in this thesis now form the basis for further studies to identify subpopulations of cells that represent specific targets for therapy, and to investigate the effects of different treatment modalities.

Key words: tumor microenvironment, double hypoxic marker method, hypoxia, hypoxic half-life, and proliferation.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCON</td>
<td>accelerated radiotherapy with carbogen and nicotinamide</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>carbogen</td>
<td>95% O₂ + 5% CO₂</td>
</tr>
<tr>
<td>CCI-103F</td>
<td>1-(2-hydroxy-3-hexafluoroisopropoxy-propyl)-2-nitroimidazole</td>
</tr>
<tr>
<td>DiOC7₁</td>
<td>3,3-diheptyloxyxycarbocyanine</td>
</tr>
<tr>
<td>EF5</td>
<td>2-(2-nitro-1h-imidazol-1-yl)-n-(2,2,3,3,3-pentafluoropropyl) acetamide</td>
</tr>
<tr>
<td>EIH</td>
<td>elongation index of hypoxia</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>glut-1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>glut-3</td>
<td>glucose transporter 3</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosine staining</td>
</tr>
<tr>
<td>HbO₂</td>
<td>intravascular blood oxygen saturations</td>
</tr>
<tr>
<td>HF</td>
<td>hypoxic fraction</td>
</tr>
<tr>
<td>hif-1</td>
<td>hypoxic inducible factor 1</td>
</tr>
<tr>
<td>hoechst 33342</td>
<td>bisbenzamide fluorochrome hoechst 33342</td>
</tr>
<tr>
<td>IdUrd</td>
<td>iododeoxyuridine</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>iv</td>
<td>intravenously</td>
</tr>
<tr>
<td>IVD</td>
<td>inter-vascular distances</td>
</tr>
<tr>
<td>LI</td>
<td>labeling index</td>
</tr>
<tr>
<td>NITP</td>
<td>7-(4’-(2-nitroimidazole-1-yl)-butyl)-theofylline</td>
</tr>
<tr>
<td>OER</td>
<td>oxygen enhancement ratio</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PF</td>
<td>perfused fraction</td>
</tr>
<tr>
<td>pimonidazole</td>
<td>1-[(2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride</td>
</tr>
<tr>
<td>pO₂</td>
<td>oxygen tension</td>
</tr>
<tr>
<td>T-pot</td>
<td>Potential tumor doubling time</td>
</tr>
<tr>
<td>TPZ</td>
<td>Tirapazamine</td>
</tr>
<tr>
<td>VAMP</td>
<td>vascular architecture and microenvironmental parameters</td>
</tr>
<tr>
<td>vd</td>
<td>vascular density</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS

This thesis is based on the following papers, which in text will be referred to by their roman numerals.


The original papers have been reproduced with permission from the publishers.
INTRODUCTION

General background

Solid tumors are the end result of a multi step process that transforms normal cells into a tumor. The process begins with a series of mutations resulting in neoplastic cells that respond abnormally to signals regulating cell growth and death. Further growth of these cells may result in avascular cell aggregates that are provided with oxygen and nutrients by simple diffusion from the surrounding tissue. However, growth beyond 1-2 mm diameter requires the ability to induce formation of tumor stroma and endothelial cells, a process known as angiogenesis (Folkman, 1976). In normal tissues the vascular bed is regularly organized and able to respond to the energy demand via feedback mechanisms. Therefore, cells in normal tissues rarely experience insufficient oxygen and nutrient levels. In contrast, the blood vasculature formed by tumor-induced angiogenesis is poorly organized and has a heterogeneous architecture (Denekamp, 1992; Konerding et al., 1995; Vaupel et al., 1989), which may result in an inadequate tissue oxygenation as well as in an altered pH and bio-energetic status (Höckel & Vaupel, 2001a; Vaupel et al., 1989). The proliferation rate in regions near the tumor vasculature is often high. The tumor cell proliferation rate in these regions may be stimulated by growth factors secreted by hypoxic tumor cells situated further down the oxygen gradient. These characteristic features of the tumor microenvironment are believed to cause treatment resistance and favor tumor progression into a more malignant phenotype, but can also be used as specific target for cancer treatment.

It has been known for decades that hypoxic tumor cells are more radioresistant than well-oxygenated cells, and hypoxic cells are found in most solid tumors. The negative effect of hypoxia has been confirmed in clinical studies where pretreatment oxygen tension (pO$_2$) values were predictive for local tumor control or survival after radiotherapy in advanced cancer of the uterine cervix and in lymph node metastasis of head and neck cancer (Gatenby et al., 1988, Brizel, 1997; Höckel et al., 1993; Nordsmark et al., 1996). Different strategies have been developed to increase the radiosensitivity of hypoxic cells including the use of hypoxic cell sensitizers (such as misonidazole and nimorazole), or increasing the tumor oxygenation with vasoactive drugs (such as nicotinamide), or high oxygen gas breathing at normo- or hyper-baric conditions (Henk & Smith, 1977; Kaanders et al., 1998; Overgaard et al., 1998). Accelerated Radiotherapy combined with Carbogen (95%O$_2$ and 5%CO$_2$) and
Nicotinamide (ARCON) resulted in significant radio-sensitization in the mouse mammary carcinoma tumor line CaNT (Rojas et al., 1996). Accelerated radiotherapy targets tumor cell repopulation during the course of treatment, while carbogen and nicotinamide increases the oxygenation status of tumors to reduce hypoxia-mediated radioresistance. In a phase II study ARCON has resulted in a local control rate of 80% at 3 years for 100 patients with advanced laryngeal carcinoma, which was better than any previous report for these advanced tumors (Kaanders et al., 2002). In addition, hypoxic cells can be selectively targeted by the use of bio-reductive cytotoxins such as tirapazamine (Brown & Siim, 1996).

Flow-cytometric studies have been used to study the lifetime of hypoxic cells. By using consecutive injections of two thymidine analogues it was shown in one cell line that the hypoxic lifetime was dependent on the tumor microenvironment. The hypoxic lifetime varied from 3-5 days in spheroids in vitro to 4-10 days in vivo in xenografts (Durand & Sham, 1998). Pre-treatment differences in the dynamics of proliferating or hypoxic tumor cell populations may account for varying efficiencies of different tumor specific treatment strategies. Optimal combinations of treatment modalities with different modes of action may result in complementary cell kill such as treatment strategies to increase pO₂ during radiation treatment combined with a hypoxic cytotoxin to kill residual hypoxic tumor cells. These treatment modalities alter the physiological balance between proliferating and hypoxic cell populations. Therefore it is important to understand how dynamic processes of the microenvironment in non-treated tumors affect the efficacy of different reatment modalities to enable the design and optimization of new treatment combinations.

This thesis is focused on the spatial organization of the tumor microenvironment and the dynamics of the hypoxic cell population in tumors with different micro-environmental characteristics. The dynamics of hypoxic cells in non-treated tumors as well as in tumors treated with the high oxygen content gas carbogen or the hypoxic cell cytotoxin tirapazamine were studied with a newly developed double hypoxic marker assay.

**Tumor microenvironment**

A poorly organized, heterogeneous vascular bed is one of the characteristics of the tumor microenvironment. The blood vessels have a reduced functionality resulting in a regional heterogeneity of tumor cell proliferation and hypoxia. The tumor microenvironment plays a critical role in malignant tumor progression and treatment.
resistance. The following sections cover the most important aspects of the different components of the tumor microenvironment. Since the focus of this thesis is on tumor hypoxia this will be covered most extensively.

**Tumor vasculature**

**Organization**

As mentioned above, tumor growth beyond 1-2 mm requires supply of oxygen and nutrients via blood vessels. The vasculature also drains the tumor of toxic catabolites and provides a route for tumor cells to escape the primary tumor mass and form distant metastases.

The tumor vasculature is a mixture of pre-existing normal blood vessels, and newly formed immature blood vessels formed by angiogenesis. Tumor cells that have outgrown the oxygen and nutrient supply may induce this process, the neo-vasculature is often formed from the venous side (Vaupel et al., 1989; Vaupel et al., 2001).

The resulting tumor vasculature is often heterogeneously distributed throughout the tumor tissue and the neo-vasculature generally lacks smooth muscle cells. Tumor vessels are often dilated, tortuous, elongated, and saccular. Arterio-venous shunts and branching with blind endings also occur frequently. The vessels may have an incomplete, or may even lack an endothelial lining, which often results in leaky vessels (Vaupel et al., 1989; Vaupel et al., 2001). These characteristic properties of the tumor vasculature have been shown in three-dimensional studies with corrosion casting (Steinberg et al., 1990). The intervascular distances are generally larger in tumors than in normal tissue.

It has been shown that solid tumors may contain subpopulations of tumor cells that are more or less dependent on the proximity to the vasculature. Tumor cells isolated from the distant cell population from the vasculature in a xenograft line had a better take rate in nude mice than cells originating from a cell population that were more proximal to the tumor vasculature. The tumors of the distant sub line grew faster and had a lower vascular density compared to the proximal sub line (Yu et al., 2001).
INTRODUCTION

Figure 1. The left panel shows the regular organization of the vasculature in normal colon and the right panel shows the heterogeneous and irregular organization of the vasculature in a melanoma xenograft (Konerding et al., 1998).

Prognostic value

Both the quantity and quality of the vasculature affects the tumor microenvironment. These characteristics may affect the response to different anti-cancer treatments, such as irradiation due to reduced oxygenation status or poor drug delivery (chemotherapy).

Numerous studies have indicated that vascular density is a prognostic factor for human cancers, but conflicting results have been reported both for metastatic potential and local tumor control (Aebersold et al., 2000; Albo et al., 1994; Dray et al., 1995; Gasparini et al., 1993; Janot et al., 1996; Jenssen et al., 1996; Leedy et al., 1994; Moriyama et al., 1997; Murray et al., 1997; Salven et al., 1997; Sarbia et al., 1996; Shpitzer et al., 1996; Zätterström et al., 1995). A recent study showed a U-shaped relation between microvascular density and survival in head and neck squamous cell carcinomas. Both a very low (poor oxygenation and drug availability) and a very high (intensified angiogenesis) vascular density correlated with poor prognosis after chemoradiotherapy (Koukourakis et al., 2000). These results may (at least partially) explain the conflicting data with respect to the correlation between vascular density and treatment outcome.

Vascular targeting

Tumor growth is dependent on the formation of new blood vessels through angiogenesis. This process only occurs in tumors, normal wound healing and the menstrual cycle. Angiogenesis may be induced by tumor cells that express mutant oncogenes or non-mutated proto-oncogenes (Kerbel et al., 1998).
INTRODUCTION

Around each tumor vessel thousands of cells are situated, that are dependent on the supply of oxygen and nutrient supply provided by that vessel. Theoretically, inhibition of angiogenesis could stop tumor growth while damage of existing endothelial cells (vascular targeting) may result in massive tumor cell death.

Angiogenesis inhibitors have been pointed out as a treatment target, but there are several growth factors that stimulate angiogenesis, and targeting only one of these may result in treatment resistance (Kerbel et al., 2001). Drugs directed against angiogenesis only attack the newly formed immature vasculature, and not the pre-existing mature vessels in the tumors. Therefore anti-angiogenic therapy should be carried out in combination with treatment against the existing mature vessels (vascular targeting) to be effective. Even if a combination of anti-angiogenic therapy and vascular targeting would be fully successful, still a viable piece of tumor would remain that is presumed to be fed by the normal tissue vasculature surrounding the tumor. These normal vessels are generally less responsive to vascular targeting agents (VTAs). Vascular targeting generally results in a central necrosis of cells that are often hypoxic and more radioresistant than peripheral tumor cells. Therefore radiation treatment is likely to benefit from a combination with vascular targeting.

Perfusion

In experimental and xenografted human tumors two markers, the bisbenzamide fluorochrome Hoechst 33342 (Olive et al., 1985; Smith et al., 1988) and carbocyanine dye 3,3-diheptyloxy carbocyanine (DiOC7(3)) (Olive & Durand, 1987; Trotter et al., 1989b), are available for determining the fraction of perfused vessels at the microscopic level. For patients such markers cannot be used due to carcinogenicity. It was shown that simultaneous injection of the two markers Hoechst 33342 and DiOC73 resulted in a match, while an injection interval of 20 min resulted in a partial mismatch of perfused vessels (Trotter et al., 1989a). This provided evidence for intermittent closure and opening of tumor vessels. A similar study with lectins supported the presence of transient tumor perfusion as well (Debbage et al., 1998). In contrast, a study on melanoma xenografts using Hoechst 33342 and DiOC73 did not show the presence of transient perfusion (Tufto & Rofstad, 1995).

In a dorsal skin flap window chamber it was shown that fluctuations in tumor blood flow could involve both individual vessels and clusters of vessels (Dewhirst et al., 1996). Regional fluctuations of red blood cell velocity have been detected in human tumors with laser doppler electrodes (Pigott et al., 1996). Mismatch
experiments in combination with cell sorting techniques have shown that only a small proportion of the tumor vessels experience complete cessation or reopening, but that a large proportion of the vasculature exhibited fluctuations in the perfusion rate (Durand & LePard, 1995; Durand & LePard, 1997). This was confirmed in recent studies suggesting that diffusion-limited, chronic, hypoxia may be present in the direct vicinity of blood vessels, and could result from tumor blood vessels that have plasma flow but very low or absent red blood cell flux (Dewhirst et al., 1996). This was supported by the finding of steep longitudinal gradients of intra vascular pO₂ levels (Dewhirst et al., 1999).

Immunohistochemical studies on human tumors grown as xenografts have shown that blood perfusion is heterogeneous throughout the tumor sections and that the perfusion fraction ranges from 20 to 85% (Bernsen et al., 1995; Bussink et al., 1999; Rijken et al., 2000). The heterogeneities in vascular architecture and blood perfusion may cause hypoxia and nutritional starvation in the tumor tissue.

**Proliferation**

Tumor growth is the net-effect of an altered homeostasis, with a high tumor cell proliferation that is not balanced with a similar rate of cell death. Proliferating cells can be detected with different exogenous and endogenous cell markers.

Both the PCNA and Ki67 proteins can be detected in all cell cycle phases. The PCNA gene is constitutively transcribed in all cells, but the PCNA mRNA is only detectable in proliferating cells due to increased stability in these cells (Chang et al., 1990). The highest levels of PCNA are detected during S-phase, and the most intensively stained cells may be used to determine the S-phase fraction. The proliferation marker Ki67 is also detected in the nucleus during all phases of the cell cycle. In contrast, the synthetic and non-radioactive thymidine analogues, bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) compete with the endogenous thymidine for a place in the DNA chain during DNA synthesis (Ellwart & Dörmer, 1985). BrdUrd and IdUrd are frequently used to estimate tumor cell proliferation in vivo (Wilson et al., 1988). Since the in vivo plasma half-life of BrdUrd or IdUrd is short (15 minutes), only cells that are in S-phase immediately after administration of these markers incorporate them in the DNA (Grätzner, 1982). Figure 2 shows a schematic drawing of where in the cell cycle the different proliferation markers can be detected.

Antibodies have been developed against BrdUrd and IdUrd and these substances can be detected either by flow cytometry or immunohistochemistry (Grätzner, 1982).
Multiple staining protocols on tissue sections allow analysis of proliferating cells in relation to other microenvironmental parameters such as vasculature and hypoxia (Bennett et al., 1992). Several studies have shown that proliferating cells can be visualized and analyzed qualitatively or quantitatively in relation to tumor vasculature, perfusion and hypoxia in tumor sections (Bussink et al., 1998; Kennedy et al., 1997; Rijken et al., 2000; Rijken et al., 2002; Varia et al., 1998; Wijffels et al., 2001; Wijffels et al., 2000).

The immunohistochemical analysis allows analysis of tumor sections at high spatial resolution, but it is more time consuming than flowcytometry. Although flow-cytometry allows a large number of cells to be analyzed in a short period of time, it lacks any spatial information of the tumor microenvironment. In addition to calculations of the labeling index (LI), flow-cytometry can be used to determine the duration of the S-phase (Ts) and the potential tumor volume doubling time from a single sample (T-pot) (Begg et al., 1985).

**Proliferation patterns and prognosis**

A high tumor cell proliferation rate has been shown to occur in the vicinity of tumor vasculature and decreases rapidly with increasing distance from the blood vessels. This was probably due to reduced oxygen tension and lack of nutrients (Bussink et al., 1998; Hirst & Denekamp, 1979; Tannock, 1968). It is likely that proliferating cells experiencing transient reductions in tumor oxygenation stop proliferating but can be recruited back into the cell cycle upon re-oxygenation, while cells at extreme and prolonged hypoxia are doomed to die.

In vitro experiments have shown that proliferation may occur down to 0.2-1 mm Hg (Åmellem et al., 1994; Koch et al., 1973a; Koch et al., 1973b). This implies that cells at intermediate oxygen tensions (0.5-20 mm Hg) showing reduced radio
sensitivity still are capable of proliferation. This is supported by studies in vivo, where administration of both bio-reductive hypoxic cell markers and cell proliferation markers indicated that a small number of hypoxic cells are capable of proliferation (Kennedy et al., 1997; Varia et al., 1998; Zeman et al., 1993), although the opposite has also been shown (Durand & Raleigh, 1998). The extent of cell proliferation under hypoxic conditions is not clear but may be of therapeutic importance since it could confer increased radio-resistance, and lead to rapid repopulation between radiation fractions.

For various endogenous markers of proliferation, such as PCNA, Ki-67 and cyclins, conflicting results in the correlation with treatment outcome have been reported (Björk-Eriksson et al., 1999; Lera et al., 1998; Nylander et al., 1997; Sittel et al., 2000; Sittel et al., 1999). In a large study of 467 patients treated with radiotherapy alone, a weak correlation between treatment outcome and the proliferation status of tumor cells before the start of treatment was found in head and neck cancer (Begg et al., 1999). Although the correlation with pretreatment proliferation status was often weak, the importance of proliferation for treatment outcome is clearly illustrated by the decrease in local control when radiation treatment periods are longer than 4 to 5 weeks (Withers et al., 1988).

Tumor hypoxia

Definitions of hypoxia

Tumor hypoxia is the net result of an imbalance between the O2 supply and (metabolic) demand in stroma, endothelial cells, and the tumor cells. Several definitions of hypoxia exist, and depend on the experimental endpoint or detection methods.

Although no sharp threshold exists between normoxia and hypoxia, the literature shows that median pO2 levels below 10 mm Hg generally result in intracellular acidosis, ATP depletion, and a drop in energy supply. Oxidative phosphorylation for ATP production measured in vitro continues to 0.5-10 mmHg depending on the cell line and the experimental setup, see for review (Höckel & Vaupel, 2001b). Cell cycle progression is affected (prolonged) at 0.2-1 mm Hg, and hypoxia may induce transcriptional, posttranscriptional or posttranslational changes resulting either in cell death or malignant progression in the range 1-15 mm Hg (Höckel & Vaupel, 2001b).
INTRODUCTION

Table 1. Definitions of hypoxia.

<table>
<thead>
<tr>
<th>pO₂ (mm Hg)</th>
<th>Definition/ usage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>At sea level</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Alveolar blood</td>
<td>(Stainsby et al., 1988)</td>
</tr>
<tr>
<td>95</td>
<td>Arterial blood</td>
<td>(Stainsby et al., 1988)</td>
</tr>
<tr>
<td>40</td>
<td>Venous blood</td>
<td>(Stainsby et al., 1988)</td>
</tr>
<tr>
<td>≈24-66</td>
<td>Tissue, organ dependent</td>
<td>(Vaupel, 1990)</td>
</tr>
<tr>
<td>% &lt; 5 or</td>
<td>Typical Eppendorf resolution</td>
<td></td>
</tr>
<tr>
<td>% &lt; 2.5</td>
<td>Malignancies</td>
<td>(Vaupel, 1990)</td>
</tr>
<tr>
<td>≤ 20</td>
<td>The level below which a steep increase of radioresistance begins</td>
<td>(Höckel &amp; Vaupel, 2001b)</td>
</tr>
<tr>
<td>≈25</td>
<td>Bioreductive hypoxic markers</td>
<td>(Gross et al., 1995; Koch et al., 1995)</td>
</tr>
<tr>
<td>0.5-20</td>
<td>Intermediate hypoxia</td>
<td>(Wouters &amp; Brown, 1997)</td>
</tr>
<tr>
<td>0</td>
<td>Anoxia</td>
<td></td>
</tr>
</tbody>
</table>

Together with the evidence presented in the previous paragraph, these results indicate that cells at radiobiological levels of hypoxia may show treatment resistance, but still be capable of proliferation. Due to different definitions of hypoxia in the literature there is a risk for unintentional inconsistency of the definition of hypoxia. In the experimental results presented and discussed in this thesis hypoxia is defined by reduction of bioreductive hypoxic cell markers e.g. ≤ 10 mm Hg.

Radiobiological hypoxia

Indirect evidence of oxygen-tension as an important factor for radiation response was provided by a study of skin reactions, where pressure on the skin provided radioresistance (Schwarz, 1909). In the 1920:s it was shown that the radiation dose required to prevent hatching in ascaris eggs was three times higher during anaerobic than aerobic conditions (Holthausen, 1921). Later it was shown that occlusion of blood
INTRODUCTION

flow to tissue during radiation treatment also reduced the tissue damage (Mottram, 1924). During the following years more suggestive evidence for the effect of oxygen appeared, and a differential cell kill in peripheral and central parts of tumors was demonstrated (Mottram, 1936). Preferential damage after irradiation was seen in the marginal cells, and it was hypothesized that these cells were exposed to a better vascular supply, and consequently to higher oxygen tensions than central hypoxic cells that were radioresistant. In the 1950:s Gray et al showed that the sensitivity of mouse tumor cells was about three times larger when irradiated in a well-oxygenated medium than under anoxic conditions and that the radiosensitivity increased in tumors during oxygen breathing (Gray et al., 1953). The increased radiosensitivity caused by oxygen was later called oxygen enhancement ratio (OER), and in 1963 Powers and Tolmach provided the first quantitative evidence for the OER with the demonstration of a biphasic curve with a steep initial part and a more flat section at higher doses (Powers & Tolmach, 1963). The OER was calculated to be 2.3 (Powers & Tolmach, 1963) and at present it is assumed to range from 2.5 to 3.0.

Hypoxia and prognosis

Recently tumor hypoxia has been shown to be predictive for patients receiving radiotherapy for carcinomas of the head and neck (Brizel et al., 1997; Gatenby et al., 1988; Nordsmark et al., 1996) and the uterine cervix (Höckel et al., 1993). Several methods have been developed to overcome radio resistance due to tumor cell hypoxia. These include the use of hypoxic cell sensitizers (such as misonidazole and nimorazole), or vasoactive drugs, and high oxygen gas breathing at normo- or hyperbaric conditions (Henk & Smith, 1977; Kaanders et al., 1998; Overgaard et al., 1998). A meta-analysis of 83 randomized clinical trials comparing radiotherapy alone and radiotherapy combined with treatments aimed to modify tumor hypoxia showed that both local control and survival could be improved by reducing or specifically targeting tumor hypoxia (Overgaard & Horsman, 1996). Although oxygenation-modifying treatment protocols in general have increased both local control rate and overall survival in several patient categories, not all of the treated patients benefited from these new treatment approaches. Furthermore, these patients experienced increased acute side effects compared to conventional radiotherapy (Kaanders et al., 1995). If it was possible to select patients based on tumor oxygenation parameters it could prevent unnecessary modification or intensification of treatments for a subgroup
INTRODUCTION

of patients who are unlikely to benefit from oxygenation modifying treatment approaches.

Assays for detection of hypoxia

Several methods are available to analyze tumor cell hypoxia. The pO₂ in tissue can be measured directly with polarographic needle electrodes (Vaupel et al., 1991). During these pO₂ measurements, small amounts of oxygen are consumed excluding the possibility to measure continuously at one position in a tumor (Collingridge et al., 1997). Clinical studies with the Eppendorf electrode showed that tumors with a low pre-treatment pO₂ do worse after radiotherapy compared to tumors that were better oxygenated (Brizel et al., 1997; Nordsmark et al., 1996). Recently a fiber optic based system (OxyLite) was developed. The pO₂ measurements with this method are based on pO₂-dependent changes in the half-life of an excited luminophor at the tip of the probe. This process does not consume oxygen, but relies solely on the presence of oxygen (Collingridge et al., 1997; Young et al., 1996). The fiber-optic probe can be left at one position for a longer period of time, which makes it possible to measure both temporal pO₂-changes as well as the effect of oxygen modifying treatments such as carbogen over time in tissue (Bussink et al., 2000b; Bussink et al., 2000c).

There are two methods for calculation of radiobiological hypoxia. With clonogenic assays cell survival after irradiation under normoxic and anoxic conditions is compared (Steel, 1977; Van Putten & Kallman, 1968). The second more recently introduced comet assay is based on DNA damage induced by radiation (Olive & Durand, 1992).

Tumor cell hypoxia can also be studied with the use of bio-reductive chemical markers such as the 2-nitroimidazoles (Chapman et al., 1981; Koch et al., 1995; Long et al., 1991; Raleigh et al., 1999; Raleigh et al., 1987). These markers are bio-chemically reduced and bound in tissues at oxygen tensions below approximately 10 mm Hg (Arteel et al., 1995; Gross et al., 1995), which not necessarily represents the same subset of cells that is detected as the radiobiological hypoxic fraction (Fenton et al., 1995). Recent studies comparing pimonidazole binding with radiobiological hypoxia, suggest that the bio reductive hypoxic cell markers detect hypoxic cells having pO₂-levels corresponding with radiobiological hypoxia (Olive et al., 2000; Raleigh et al., 1999).

Antibodies have been raised against several bio-reductive markers, such as pimonidazole, EF5, NITP and CCI-103F, of which the first two are being applied in...
INTRODUCTION

clinical studies (Evans et al., 2000; Kennedy et al., 1997; Raleigh et al., 2000; Wijffels et al., 2000). The hypoxic fraction can be determined either by analysis of the fraction labeled hypoxic cells by flow cytometry (Durand & Raleigh, 1998; Hodgkiss et al., 1991) or by image analysis of microscopic sections (Bussink et al., 1999; Cline et al., 1997; Evans et al., 1995; Kennedy et al., 1997; Webster et al., 1995; Wijffels et al., 2000). By combining several immunohistochemical techniques the bio-reductive binding of hypoxic markers can be related to other micro-environmental factors of tumors such as vascular architecture, tumor blood perfusion and proliferation (Bussink et al., 1999; Cline et al., 1994; Kennedy et al., 1997).

Besides the OxyLite system, mentioned above, only a limited number of assays are available by which dynamic information of the oxygenation status of tumors can be obtained. These methods include electron paramagnetic resonance (EPR) oximetry, spin-lattice relaxation of fluorinated Magnetic Resonance agents (Mason et al., 1999) and cryospectrophotometry of intra-vascular oxyhemoglobin saturation profiles (Fenton et al., 1999). With a double hypoxic cell marker technique, based on scintillation counting, a reduction of tumor hypoxia after carbogen breathing and nicotinamide administration was demonstrated (Iyer et al., 1998). A disadvantage of all these methods is the poor spatial resolution; therefore changes of the tumor oxygenation status cannot be related to other micro-environmental parameters.

In this thesis a double hypoxic marker method will be presented that can be used to detect changes in hypoxia, with high spatial resolution, relative to the tissue architecture.

Patho-physiology of hypoxia

In the 1950:s a corded tumor structure was described, where endothelial stroma surrounded the cords that enclosed a central region of tumor necrosis (Thomlinson & Gray, 1955). It was concluded that the necrosis was caused by diffusion-limited starvation, and that the crucial nutrient in this process was oxygen. Based on early data of oxygen consumption rates, Thomlinson and Gray postulated that the expected oxygen diffusion distance in tissue would be approximately 150 µm, and the measured thickness of the viable tumor cell rims never exceeded 180 µm (Thomlinson & Gray, 1955). Later an inverted structure of tumor tissue with tumor cords was described that contains a central blood vessel, surrounded by a rim of viable tumor cells, followed by tumor necrosis at larger distances from the vessels. The radius of the viable tumor cords generally ranged from 60-120 µm (Tannock, 1968), and classical corded tumors
described in the literature generally have the histological structure that was described by Tannock (1968). Figure 3 shows the differences between the two types of corded tumors.

![Tumor cord diagram](image)

**Figure 3.** Schematical drawing of the two tumor cords described in the literature (Tannock, 1968; Thomlinson & Gray, 1955). N= necrosis

Both types of corded tumor structures show diffusion limited hypoxia occurring at increasing distance from the tumor vasculature, a phenomenon called chronic hypoxia. Later it was shown that hypoxia also could occur in the direct vicinity of blood vessels that may be caused by transient opening and closure of tumor vessels (Brown, 1979). The above mentioned two forms of hypoxia, chronic, diffusion limited and acute, perfusion limited hypoxia are shown in figure 4. This is a simplification of a complex situation, and confers the extreme cases of a more dynamic situation. Recent studies suggest that complete occlusion of vasculature is rather uncommon, but small changes of the perfusion rate may occur frequently (Dewhirst et al., 1996). These changes can result in very low or absent red blood cell flux rate resulting in steep longitudinal gradients of oxygen and nutrients along vessels and may causing transient periods of hypoxia (Dewhirst et al., 1999; Walenta et al., 2001).

Nutrients with longer diffusion distances than oxygen such as glucose may provide chronically hypoxic cells with enough energy go into a state of anaerobic metabolism (Vaupel, 1990). Therefore these cells may remain viable for prolonged periods of times.
**Dynamics of hypoxic cells**

The tumor microenvironment is constantly changing due to tumor growth as well as temporal and spatial variations in blood supply. This results in regional changes in hypoxia, apoptosis and proliferation rate within a tumor. Considering the heterogeneity of the tumor vasculature and amount of hypoxia it is likely that there are large differences in the lifetime of hypoxic cells.

In vitro studies showed that the life time of hypoxic cells could extend to several days under severe hypoxic conditions (Koch et al., 1973a; Littbrand & Revesz, 1968). Later it became evident that the inner, nutrient deprived cells in V79 spheroids were more vulnerable to acute hypoxia than the more viable outer cells (Franko & Sutherland, 1978). The hypoxic cells in the center of a spheroid died within 4 h, but in the rim of a spheroid cells died after more than 6 h. Cell loss in human tumors occurs predominantly in the nutrient and oxygen deprived cell compartment. It may be assumed that the lifetime of hypoxic cells depends on various factors such as for instance cell turnover from any labeled cell cohort of known age, such as a population that is pulse labeled with IdUrd. Based on this assumption the turnover rate of cells labeled with IdUrd was used to estimate the hypoxic cell lifetime in the SiHa tumor line. In the spheroids from this line the hypoxic lifetime was 3-5 days, while in the xenografts this was 4-10 days (Durand & Sham, 1998). From these studies it was concluded that the lifetime of hypoxic cells depends on the tumor microenvironment (Durand & Sham, 1998).

According to the theory by Thomlinson and Gray, new cells were formed in the proliferating cell compartment that consequently pushed cells away from the vessel and
INTRODUCTION

exposed the cells to a gradual decrease of oxygenation and nutrients. Cells in the hypoxic cell compartment would be pushed further down the oxygen gradient and eventually die of oxygen deficiency and starvation. According to this theory the proliferation rate would determine the hypoxic cell turnover rate.

Normal cells that become hypoxic induce angiogenesis and start a suicidal program leading to apoptosis if the hypoxia is severe enough. However if tumor cells have adapted to the harsh hypoxic environment they may still produce angiogenic factors, combined with an acquired ability to survive under hypoxic conditions and remain viable for a longer period of time (Höckel & Vaupel, 2001a; Schmaltz et al., 1998; Vaupel & Hockel, 2003; Wouters et al., 2003). An in vitro study has shown that the mechanism of apoptosis induced by hypoxia seems to require acidosis and that the cells were rescued by enhanced buffering. When uncoupled from acidosis hypoxia was shown to enhance tumor cell viability and clonogenicity relative to normoxia due to down regulation of p53 (Schmaltz et al., 1998). This suggests that the viability and lifetime of hypoxic cells may depend on their response to the tumor microenvironment.

Hypoxia and malignant progression

As mentioned above, tumor hypoxia may induce genetic changes, cell quiescence, differentiation, apoptosis, or necrosis. Generally, extreme hypoxia leads to G1/S phase cell cycle arrest, and below a critical energy state hypoxia may cause necrotic cell death. Moderate hypoxia may induce apoptosis via the p53 pathway or the p53-independent hypoxia inducible factor 1 (Hif-1α) pathway. In addition to induced cell death, hypoxia may also promote cell growth and malignant progression by adaptation to nutritional starvation. A large set of molecules and transcription factors is induced by hypoxia and has been related to tumor progression. Extended periods of tumor hypoxia results in increased genomic instability that promotes tumor cell adaptation to the hostile microenvironment. Clonal selection and expansion of these cells may lead to tumor propagation and formation of new hypoxic cells, followed by further adaptation and clonal selection resulting in a vicious circle for review see (Höckel & Vaupel, 2001b).

Among the genes induced by hypoxia are glycolytic enzymes, glucose transporters (glut-1 and glut-3) that facilitate anaerobic metabolism and carbonic anhydrase IX (CA9) to maintain a constant intracellular pH level. These factors are regulated via the von Hippel-Lindau tumor suppressor protein and Hif-1α pathway.
Glut-1, CA9 and Hif-1α expression is often found at increasing distance from blood vessels, suggesting that they could be a marker for diffusion-limited hypoxia.

Hif-1α helps to restore oxygen homeostasis by induction of glycolysis, erythropoiesis and angiogenesis. Hif1-α is mainly found near necrotic areas, suggesting a relation with diffusion limited hypoxia, although expression also is found close to blood vessels (Aebersold et al., 2001; Talks et al., 2000; Vukovic et al., 2001; Zagzag et al., 2000; Zhong et al., 1999). Expression of HIF-1α is found closer to blood vessels than bio-reductive hypoxic cell markers which is suggestive for upregulation at higher pO2-levels than at which reduction of agents such as pimonidazole and EF5 take place (Vukovic et al., 2001). In oropharynx tumors the expression of Hif-1α was reported to correlate with loco-regional control after radiotherapy (Aebersold et al., 2001).

The carbonic anhydrases are involved in maintaining intracellular pH and over-expression in tumor cells causes a reduction of the extracellular pH thereby facilitating the breakdown of the extracellular matrix (Giatromanolaki et al., 2001). These mechanisms may play an important role towards a more aggressive tumor behavior and increased metastatic potential. A steep increase of CA9 expression was demonstrated during 4 to 24 h after hypoxic induction and the expression of CA9 was shown to occur at pO2 levels below 20 mmHg (Wykoff et al., 2000). Several studies found a correlation between pimonidazole binding and CA9 expression, and generally the tissue area showing CA9 expression was larger than the pimonidazole-positive area (Lal et al., 2001; Olive et al., 2001; Wykoff et al., 2001). The areas of mismatch may reflect differences in oxygenation levels at which the two markers are induced, but it may also reflect existence of temporal changes in hypoxia (Olive et al., 2001). Expression of CA9 correlates with poor outcome in several tumor types such as carcinoma of the breast, non-small lung cancer and carcinoma of the cervix (Giatromanolaki et al., 2001; Loncaster et al., 2001; Wykoff et al., 2001). Correlation with outcome has also been investigated for the glucose transporters and a weak but significant correlation between Glut-1 expression and pO2 measurements in advanced cancers of the uterine cervix was found (Airley et al., 2001).

Tumor hypoxia induces many processes via Hif-1α such as reduction of proliferation rate and increase in apoptosis (Akakura et al., 2001; Carmeliet et al., 1998). Several studies show that there is a relationship between hypoxia and Hif-1α, but because it is an upstream transcription factor that regulates several pathways it may not be the ideal surrogate marker for diffusion limited hypoxia.
INTRODUCTION

Cell loss, apoptosis and necrosis

Cell loss may occur through apoptosis induced by hypoxic cells that experience intermediate hypoxia. In contrast, necrosis occurs under extreme hypoxia, when the energy supply in the tumor cells is too low to be able to initiate apoptosis. On frozen H&E stained tissue sections it is not easy to distinguish apoptosis from necrosis and in this thesis, large confluent areas of cell death are denoted as being necrotic. Apoptotic cells can be detected with antibodies against proteins involved in the apoptotic pathway such as the TUNEL method or staining of activated caspase-3 (Gavrieli et al., 1992; Krajewska et al., 1997).

Alterations of genes involved in apoptosis are important for malignant progression and for treatment outcome (Reed, 1999), and tumor hypoxia in combination with a low apoptotic index predicts for poor survival in carcinomas of the uterine cervix (Höckel et al., 1999). The relationship between apoptosis and treatment outcome depends on the histological tumor type. Adenocarcinomas of the uterine cervix with a high apoptotic index have a good prognosis after treatment (Sheridan et al., 1999; Wheeler et al., 1995) while squamous cell carcinomas of the same site with a high apoptotic index have a poor prognosis (Levine et al., 1995; Tsang et al., 1999a; Tsang et al., 1999b). The relationship between hypoxia and apoptosis suggests that tumor reoxygenation during radiotherapy could partially be explained by cell death via apoptosis in adenocarcinomas of the cervix (Sheridan et al., 2000).

Relation between proliferation, hypoxia and cell loss

From the preceding paragraphs it is obvious that although the different microenvironmental parameters are correlated with treatment outcome it is a reflection of a dynamic environment. Single parameter analysis shows that there are large inter tumor heterogeneities in all studied parameters. By combining these into a multi parameter profile, the interplay between these factors can be analyzed. It is likely that the dynamic interactions between different cell populations provide information of why some tumors respond well to treatments targeting specific subpopulations in the tumor cells, while others don’t.

Cytotoxic treatments alter the complex tumor microenvironment. After irradiation, pO$_2$ in tumors increases due to a decreased oxygen consumption and alteration of blood supply. The decrease in consumption is due to a reduction in the number of respiring cells as a result of cell cycle arrest, apoptosis and necrosis. This direct effect of irradiation causes shrinkage of the tumor and leads to a decrease of the
hydrostatic pressure that eventually results in an improved blood supply (Kallman & Dorie, 1986). In experimental tumors an improvement of the oxygenation status was found with maximum oxygenation several hours after irradiation (Bussink et al., 2000a; Fenton, 1997; Olive, 1994). Analysis of the temporal changes of hypoxia in a human xenografted head and neck squamous cell carcinoma tumor line, revealed that 3-6 days post-irradiation, hypoxia had returned to the pretreatment level (Bussink et al., 2000a). The initial reduction of tumor hypoxia could not be explained by an increase in tumor blood perfusion but was most likely due to a decrease in oxygen consumption (Bussink et al., 2000a; Olive, 1994). For cancers of the uterine cervix it was shown that changes in hypoxia in the early phase of fractionated radiotherapy depend on the balance between changes in cell density and vascular damage such as endothelial swelling and hypertrophy (Lyng et al., 2000).

The above-mentioned studies provide only limited quantitative insight into the actual spatial and temporal changes in hypoxic cell populations induced by cytotoxic treatments, but they clearly show the need to understand how these treatments affect tumors with different microenvironmental characteristics and dynamics of hypoxic cells, in order to design optimal treatment schedules aimed at complementary cell kill of different tumor cell populations.
AIMS OF THE PRESENT STUDY

This thesis is focused on the dynamic relationships of tumor cells within the tumor microenvironment. Tumor vasculature, hypoxia, and proliferation was visualized by triple stainings of frozen tissue sections and analyzed with image analysis techniques.

The specific aims of this thesis are to:

I. Characterize the physiological states of a panel of human tumors, grown as xenografts, based on the vascular architecture, distribution of hypoxic cells, and the tumor cell proliferation rate.

   A novel double hypoxic marker method was developed:

II. To determine the half-life of hypoxic tumor cells in three tumor lines that differ in their hypoxic cell organization with the double hypoxic marker method.

III. To study dynamic changes of the hypoxic cell population in relation to the functional vasculature upon oxygen modifying treatments.

IV. To determine the effect of the hypoxic cytotoxin tirapazamine on the oxygenation status during the first 48 hours after administration of the drug.
Tumor models

The majority of the experiments were performed on human head and neck squamous cell carcinomas grown as xenografts in nude mice (Balb/c nu/nu mouse) (all papers). In paper III also the tumor lines MEC82 and C38 were used. MEC82 is a human head and neck xenograft line derived from mucoepidermoid cell carcinoma of the mouth floor grown in nude mice. C38 is a murine colon tumor line transplanted in C75BL/6 black mice (Peters et al., 1987). The tumor lines were maintained by serial transplantation of a small (1-2 mm³) piece of tumor on the right flank into 5 recipients.

In all experiments tumors with a mean diameter of 6-8 mm were used. The tumors were measured with a caliper and the volume was calculated from three orthogonal diameters according to the formula: tumor volume (mm³)=XxYxZx(π/6) (Dethlefsen et al., 1968).

The nude mice were kept in a specific-pathogen-free unit in accordance with institutional guidelines. The experiments with cytotoxins were performed at the Department of Toxicology of the Central Animal Laboratory. Approval of the local ethical committee for animal use was obtained for all experiments.

Exogenous cell markers and administered drugs

As markers of hypoxia, CCI-103F (1-(2-hydroxy-3-hexafluoroisopropoxy-propyl)-2-nitroimidazole) (Raleigh et al., 1986; Raleigh et al., 1987) and pimonidazole (1-[2-hydroxy-3-piperidinyl]propyl]-2-nitroimidazole hydrochloride) (Raleigh et al., 1995; Zeman et al., 1993) were used. CCI-103F and pimonidazole hydrochloride are bioreductive chemical probes, which only differ in the immuno-recognisable side chain (Figure 5).
The addition of the first electron in the bioreductive activation is reversibly inhibited by oxygen futile cycling with a half maximal pO$_2$ of inhibition of about 3 mm Hg, and with complete inhibition occurring below 10 mm Hg (Gross et al., 1995). Although the pharmaco-kinetics and the partition coefficients are different for the two markers the oxygen dependence of CCI-103F and pimonidazole has been shown to be consistent with misonidazole binding (Arteel et al., 1995; Raleigh et al., 1987). Pimonidazole accumulates with a tumor to plasma ratio of 3.0 (Saunders et al., 1984) and CCI-103F with a tumor to plasma ratio of 0.8 (Raleigh et al., 1986). The plasma half-life in mice is about 30 min for pimonidazole and about 41 to 90 min CCI-103F (Jin et al., 1990; Raleigh et al., 1999; Raleigh et al., 1986). This taken together makes it likely that the absolute amounts of the reduced drugs in the individual tumor cells may differ, but that the binding profiles follow the same pattern. Figure 6 shows an example of binding the profiles for pimonidazole and CCI-103F the SCCNij3 tumor line.

Pimonidazole was administered intravenously (iv) at a dose of 80 mg/kg in a volume of 0.1 ml in saline. CCI-103F was dissolved in 10% dimethyl sulfoxide (DMSO) and 90% peanut oil and was given at a dose of 80 mg/kg intraperitoneally (ip) in a total volume of 0.5 ml. The antibodies that have been raised against these two hypoxic markers did not show any cross reactivity (Arteel et al., 1995; Azuma et al., 1997; Cline et al., 1990; Raleigh et al., 1987).

The S-phase markers bromodeoxyuridine (BrdUrd, Sigma, St Louis, MO) was given ip at a dose of 25 mg/ml in a volume of 0.5 ml saline. As marker of tumor blood perfusion the fluorescent dye Hoechst 33342 (Sigma), dissolved in saline, was given iv.
at a dose of 15 mg/kg in a volume of 0.1 ml. Hoechst 33342 is cleared from the bloodstream in mice with a half live of 110 s, and is therefore only available for DNA binding during the first few minutes after administration (Olive et al., 1985). Hoechst 33342 binds to the nuclei of cells adjacent to perfused endothelial cells.

Oxygen modifying agents: For carbogen breathing (95% O₂, 5% CO₂), animals were placed in a perspex box with a continuous flow of carbogen at 5-7 l/min. The vasodilator hydralazine (Sigma) was dissolved in saline and injected iv in the tail vein at a dose of 10 mg/kg in a total volume of 0.1 ml. Hydralazine causes an acute vasodilatation of the vascular bed in the normal tissue surrounding the tumor. Thereby the blood perfusion is rapidly decreased in the tumor, resulting in an acute decrease of the oxygen delivery to the tumor tissue.

Cytotoxic agents: The hypoxic cytotoxin tirapazamine (TPZ, Sanofi, Netherlands) was dissolved in saline and given iv in the tail vein at a dose of 36 mg/kg in a total volume of 0.1 ml.

The above mentioned exogenous cell markers and drugs were administered in different combination and time schedules depending on the assays. Table 2 shows the combinations that were used in the different papers. For further details of the injection sequences see the individual papers.
MATERIAL AND METHODS

Table 2. Combinations of cell markers and drugs

<table>
<thead>
<tr>
<th>Marker</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCI-103F</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pimonidazole</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Carbogen</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Hydralazine</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Tirapazamine</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

At the end of the experiment the tumor specimens were removed and directly stored in liquid nitrogen until cut into frozen sections. Consecutive tissue sections were cut at the largest circumference of the tumor, and stored at –80°C until further processed.

Immunohistochemistry

After thawing the sections were fixed in aceton. The Hoechst signal was scanned dry prior to any immunohistochemical staining. Then the slides were hydrated in phosphate buffered saline (PBS) 0.1 M pH 7.4 (paper I and III) or in polyclonal liquid diluent immunostain PLD (DPC Breda Diagnostic Products Corporation) (paper II and IV). Between all consecutive steps of the staining procedures the sections were rinsed three times for two minutes in PBS. The antibodies were diluted in PBS with 0.1% Bovine Serum Albumin c and 0.1% Tween (PBS-BT) in paper I and III, and with PLD in paper II and IV. The endothelium was always stained in combination with one or two other cell markers. The combinations stained in the different papers are indicated in Table 3.
Table 3. Staining combinations used in the different papers

<table>
<thead>
<tr>
<th>(Perfusion Hoechst)*</th>
<th>Endothelium</th>
<th>Hypoxia pimonidazole</th>
<th>Hypoxia CCI-103F</th>
<th>Proliferation BrdUrd</th>
<th>Additional markers</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>I / III</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>I</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>III</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>II / IV</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>IV</td>
</tr>
</tbody>
</table>

* Auto-fluorescent, scanned dry prior to immunohistochemical staining.

Depending on the combinations of markers visualized, different combinations of secondary and tertiary antibodies were used to prevent cross reactivity. This was always checked in control sections. For details see the different studies. In addition the staining intensities were adjusted to the dynamic ranges of the CCD cameras of the two image analysis systems. The following primary antibodies were used in this study:

- **Endothelium**: 9F1, rat monoclonal antibody (Mab), Department of Pathology, UMC St Radboud, Nijmegen, The Netherlands, (Bussink et al., 1998; Westphal et al., 1997)
- **Pimonidazole**: rabbit anti-pimonidazole antiserum, polyclonal antibody (Pab), (Arteel et al., 1995; Azuma et al., 1997)
- **CCI-103F**: rabbit anti-CCI-103F antiserum, Pab, (Cline et al., 1990; Raleigh et al., 1987)
- **S-phase cells**: Br-3, mouse Mab to BrdUrd, Caltag laboratories, San Francisco, CA, (Bussink et al., 1998)
MATERIAL AND METHODS

Image acquisition

In paper I and III, an 8-bit image analysis system was used, and in paper II and IV a 12-bit image analysis system was used. The 8-bit system has previously been described in detail (Rijken et al., 1995). It has been used to analyze tumor cell hypoxia and proliferation (Bussink et al., 1998; Bussink et al., 1999). In Table 4 the main differences between the two systems are summarized. The newer 12-bit system has a higher resolution, and more memory capacity, and it has one extra filter.

The filters used in the two systems are listed in Table 4. The extra filter in the 12-bit system is used for staining vasculature with a fluorochrome that is barely visible to the human eye (far red), but it can easily be recorded with the CCD camera. This allows recording of up to four signals per tumor section. Another feature of the CCD camera of the 12-bit system worth mentioning is the possibility to record bright field staining. Thus it is possible to combine fluorescent and bright field microscopy in individual tumor sections. This possibility is not used in this thesis but will be developed in future studies. The scans were performed at 100X magnification for tissue sections stained for hypoxia and at 200X magnification for tissue sections stained for BrdUrd.

Resolution

The starting point of each scan (upper left) was determined and x-y stepping the coordinates were stored in the computer memory. The number of recorded fields depends on the tumor size and may be up to 12 x 12 fields (e.g. up to 176.6 mm² for the 8-bit system and up to 343.2 mm² for the 12-bit system). The tumor sections were scanned field by field in a meander pattern.

Due to the above-mentioned limitations in memory two reduction steps of 50% were performed during image recording and processing in the 8-bit system in comparison with one 50% reduction step in the 12-bit system. This results in a final resolution of 7.200 µm/pixel for the 8-bit system and 2.668 µm/pixel in the 12-bit system.
## MATERIAL AND METHODS

Table 4. Selected specifications for the two image analysis systems.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>8 bit system (Paper I/II)</th>
<th>12-bit system (Paper III/IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp</td>
<td>HBO 100</td>
<td>HBO 100 adjustable from 1-100%</td>
</tr>
<tr>
<td>Microscope (Zeiss)</td>
<td>Axisokop</td>
<td>Axioskop II</td>
</tr>
<tr>
<td>UV filter</td>
<td>Excitation 365 nm</td>
<td>Excitation 365 nm</td>
</tr>
<tr>
<td></td>
<td>Emission 420 nm</td>
<td>Emission 420 nm</td>
</tr>
<tr>
<td></td>
<td>Excitation 450-490 nm</td>
<td>Excitation 450-490 nm</td>
</tr>
<tr>
<td>Green</td>
<td>Emission 520 nm</td>
<td>Emission 515-565 nm</td>
</tr>
<tr>
<td></td>
<td>Excitation 510-560 nm</td>
<td>Excitation 546/12 nm</td>
</tr>
<tr>
<td>Red</td>
<td>Emission 690 nm</td>
<td>Emission 575-640 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission 575-625 nm</td>
</tr>
<tr>
<td>Far red</td>
<td></td>
<td>Emission 660-710 nm</td>
</tr>
<tr>
<td>Detection range CCD camera</td>
<td>Fluorescence</td>
<td>Fluorescence and bright field</td>
</tr>
<tr>
<td>Video signal</td>
<td>Analogue</td>
<td>Digital</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Manual gain control</td>
<td>Integration signal</td>
</tr>
<tr>
<td>No of images averaged to minimize noise</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Grey levels</td>
<td>256</td>
<td>4096</td>
</tr>
<tr>
<td>Resolution CCD camera</td>
<td>752 x 504 pixels</td>
<td>1300 x 1030 pixels</td>
</tr>
<tr>
<td>Resolution of the composite binary images</td>
<td>25% (Two 50% reduction steps)</td>
<td>50% (one reduction step)</td>
</tr>
<tr>
<td>$\mu$m per pixel in binary composite image at 100X</td>
<td>7.2 $\mu$m/pixel</td>
<td>2.668 $\mu$m/pixel</td>
</tr>
<tr>
<td>Maximal scan size (12 x 12 fields)</td>
<td>16.2 x 10.9 mm</td>
<td>20.8 x 16.5 mm</td>
</tr>
<tr>
<td>Software for image recording and processing</td>
<td>TCL-image (TNO, Delft, Netherlands)</td>
<td>IP-Lab (Scanalytic Inc. Fairfax, VA, USA)</td>
</tr>
<tr>
<td>Saved image mode</td>
<td>Binary</td>
<td>Gray scale + binary</td>
</tr>
<tr>
<td>Software for outlining tumor area and masks</td>
<td>TCL-image</td>
<td>IP-Lab</td>
</tr>
<tr>
<td>Quantitative analysis</td>
<td>TCL-image</td>
<td>TCL-image</td>
</tr>
</tbody>
</table>
MATERIAL AND METHODS

Scanning and thresholding, 8-bit system

The left flow-chart (page 39) shows the steps in the scanning procedure for the 8-bit system. The segmentation for each recorded signal was set in one representative field after background reduction, on which a max-min operation was applied. The thresholds were interactively set to isolate the foreground pixels from the background. The max-min filter was applied on all recorded fields. This corrected for uneven illumination and reduced small differences of the background levels of the stained cell markers both within and between tumor sections. This resulted in one relative threshold for each marker within one experiment.

For all markers studied in this thesis, except the hypoxic cell markers, the threshold was tumor line independent. Pilot experiments had shown that for tumors of different passages (first passage versus third passage) of the same xenograft tumor line one common threshold could be set per hypoxic marker (unpublished data). Different thresholds were set for the hypoxic markers CCI-103F and pimonidazole. Thus a separate threshold was set for the two hypoxic markers (pimonidazole and CCI-103F) in each tumor line.

Since the individual fields were recorded and immediately processed into a binary image without storage of the original gray level image it was not possible to change the threshold afterwards on the composite binary image.

Scanning and thresholding, 12-bit system

The right flowchart in (page 39) shows the sequence of the different steps in the scanning procedure for the 12-bit system. The scans were performed with constant light intensity and exposure time for each cell marker within one experiment. This system has more setting options than the 8-bit system that allows setting of the segmentation level (threshold) both at the beginning of a scanning session and after the scanning procedure in an already stored composite gray scale image. A background image, an illuminated clean glass slide, recorded before each scanning session was subtracted from the individual recorded fields. The thresholds for tumor vasculature
### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th><strong>8 bit system</strong></th>
<th><strong>16 bit system</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initialization and calibration of the scanning stage</td>
<td>Initialization and calibration of the scanning stage</td>
</tr>
<tr>
<td>Illumination and camera gain are set for optimal image recording</td>
<td>Illumination is set to obtain maximal dynamic range of signal intensities</td>
</tr>
<tr>
<td>One threshold value for segmenting the structures from the background is recorded in a representative field and stored for later use</td>
<td>Camera exposure time is set to obtain maximal dynamic range of signal intensities</td>
</tr>
<tr>
<td>During scanning individual fields are recorded, corrected for uneven illumination (using local min-max filter operation), binarized with the recorded threshold, and montaged into one composite binary image</td>
<td>One background image is recorded using a clean glass slide</td>
</tr>
<tr>
<td>The composite images of all signals are layered into one image for further quantitative analysis</td>
<td>Binarizing: one threshold per experiment for vasculature and perfusion; one threshold for each tumor section for the hypoxic markers.</td>
</tr>
<tr>
<td>Tumor area and masks are drawn and added as to the corresponding layered image to exclude staining artefacts and necrosis</td>
<td>The tumor area and masks are drawn to exclude staining artefacts and necrosis (binary)</td>
</tr>
<tr>
<td>The composite images of all signals are layered into one image for further quantitative analysis</td>
<td>The composite images of all signals are layered into one image for further quantitative analysis</td>
</tr>
</tbody>
</table>
MATERIAL AND METHODS

and perfusion were set once for the whole experiment in analogy with the 8-bit system. This was not possible for the hypoxic cell markers with the 12-bit system.

To allow absolute measurements of hypoxic marker binding intensities as function of distance to the vasculature, the staining intensity was adjusted to make optimal use of the dynamic range of the camera. For the same reason a max-min operation was not applied on the recorded fields. The combination of a more sensitive system and inter-tumor differences in hypoxic cell marker gradients prevents setting of a tumor-line specific absolute threshold for the hypoxic markers.

The human eye can distinguish about 255 gray levels while the 12-bit CCD camera detects 4096 gray levels, therefore it was difficult to interactively set a correct threshold of the hypoxic cell marker signal. Instead a standardized numerical method for thresholding was developed. Briefly, a gray-value histogram of the intensity values was generated from the total number of pixels in the whole recorded image. The histogram was visualized with a linear axis for the number of pixels and a logarithmic scale for the intensity values. A curve fit was calculated on the part of the histogram that showed a linear decrease in the number of pixels over a logarithmic range of intensity values. The intersection of the curve fit with the intensity values (x-axis) was calculated and used as threshold for the tumor-section. Composite binary images were then created based on the calculated segmentation value.

A consecutive H&E stained tumor section was used to create a binary image with a mask of the tumor area, excluding non-tumor tissue and necrosis from the analysis. A comparable “mask image” was defined in the same way in which the hypoxic cell markers were defined without artifacts. Thus, for each tumor scan seven binary images were generated, consisting of four scans (vasculature, perfusion marker, first and second hypoxic marker) and three masks (tumor area, first and second hypoxia masks).

Post processing of images exported from IP-Lab to TCL-image

As indicated in Table 4, different software packages were used for image acquisition and image processing. On the 12-bit system IP-Lab was used for scanning
MATERIAL AND METHODS

and creation of binary images. After generating the masks, the binary images from the scans were exported to the image analysis software TCL-image (TNO, Delft, The Netherlands). This resulted in one image with the seven different scans layered for spatial analysis of hypoxia and other tumor microenvironmental parameters.

Before performing quantitative analysis two image analysis operations were performed: 1) “dilation” of Hoechst 33342 signal with two pixels, and 2) “closing” of two pixels of the hypoxia signal.

The pixels positive for endothelial cells and Hoechst 33342 positive cells surrounding these did not overlap due to the high resolution. Therefore the first operation was performed to facilitate analysis of overlap of the vasculature and perfusion markers Hoechst 33342 e.g. the perfused vessels.

The hypoxic cell markers pimonidazole and CCI-103F are mainly detected in the cytoplasm of hypoxic cells. Tumor cells containing cross-sections of nuclei contains visible dark non-stained “holes” appear in the tumor sections. These were also present in the binary images. The second operation prevented underestimation of the hypoxic fraction caused by negative areas of cross sections of nuclei in hypoxic regions. This operation was performed for both hypoxic cell markers. It was chosen not to make a complete fill of these negative areas, to prevent that small necrotic foci accidentally would be analyzed as hypoxia. The maximal area that was completely filled was less than the size of an average mammalian cell.

Quantitative parameters:

Although the image acquisition and the post-processing of the images differed between the two systems, the quantitative parameters were identical for the image analysis systems. Table 5 shows a list of all quantitative parameters that were used in this thesis and the references for these. Considering the fact that different staining protocols were used for the two image analysis systems, which have different resolutions and different post-processing of the images, differences of the absolute values from the quantitative analysis were expected to occur.
Table 5. A list of the parameters calculated from the binary images.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation</th>
<th>Definition</th>
<th>Reference:</th>
<th>Used in paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfused fraction</td>
<td>PF</td>
<td>The area of vascular structures labeled with the perfusion marker Hoechst 33342 divided by the total vascular area.</td>
<td>(Bernsen et al., 1995; Rijken et al., 1995)</td>
<td>I / II / III</td>
</tr>
<tr>
<td>Hypoxic fraction</td>
<td>HF</td>
<td>The fraction of the tumor surface stained for the hypoxic marker relative to the total tumor surface.</td>
<td>(Bussink et al., 1999; Rijken et al., 2000)</td>
<td>All</td>
</tr>
<tr>
<td>Labeling index</td>
<td>LI</td>
<td>the BrdUrd-labeled area divided by the Fast Blue labeled area</td>
<td>(Bussink et al., 1998)</td>
<td>I</td>
</tr>
<tr>
<td>Elongation index hypoxia</td>
<td>EIH</td>
<td>The longest (A) and the shortest (B) axis of each hypoxic area was determined, and the ratio was then calculated according to A/B.</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Vascular density</td>
<td>VD</td>
<td>The number of vascular structures per mm².</td>
<td>(Bernsen et al., 1998; Rijken et al., 2000; Rijken et al., 1995)</td>
<td>I</td>
</tr>
<tr>
<td>Inter vascular distances</td>
<td>IVD</td>
<td>The shortest distance between tumor vessels (µm)</td>
<td>(Rijken et al., 2000)</td>
<td>I</td>
</tr>
<tr>
<td>HF per zone</td>
<td>HFZ</td>
<td>HF was determined in six zones at 0-22, 22-50, 50-100, 100-150, 150-200, and 200-250 µm around each vessel, and was presented as the mean of all vessels</td>
<td>(Bussink et al., 1999; Rijken et al., 2000; Wijffels et al., 2000)</td>
<td>I</td>
</tr>
<tr>
<td>LI per zone</td>
<td>LI per zone</td>
<td>The LI was analyzed in seven zones at 0-25, 25-50, 50-75, 75-100, 100-150, 150-200, and 200-250 µm around each vessel and presented as the mean of all vessels.</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Fraction non viable tissue</td>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Dynamics of hypoxia</td>
<td>HF1/HF2</td>
<td>The first hypoxic fraction divided by the second hypoxic fraction</td>
<td></td>
<td>II / IV</td>
</tr>
</tbody>
</table>
A comparison of the values for the two hypoxic markers CCI-103F and pimonidazole in the SCCNij3 line was done for two experiments (Table 6). The experiment in paper II was analyzed with help of the 12-bit system and the experiment in paper III was analyzed with help of the 8-bit system. The hypoxic fractions (HF) obtained for control tumors in the two experiments were similar, but the median HF for both pimonidazole is CCI-103F is smaller when analyzed with the 12-bit system compared with the 8-bit system. However, the differences between the systems are larger for CCI-103F than for pimonidazole, which results in a larger mismatch between the two markers on the 12-bit system. The resulting ratio is around 1.6 and corresponds to a one to maximally two cell layers difference in distance to the start of hypoxia for the two markers, in a tumor cord of about 150 µm.

Table 6. Comparison of HF in the SCCNij3 line for pimonidazole and CCI-103F for the 12-bit (n=5) and the 8-bit system (n=7).

<table>
<thead>
<tr>
<th></th>
<th>Pimonidazole</th>
<th>CCI-103F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12-bit system</td>
<td>8-bit system</td>
</tr>
<tr>
<td>Median</td>
<td>6.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Mean</td>
<td>10.6</td>
<td>7.0</td>
</tr>
<tr>
<td>SEM</td>
<td>4.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>
SUMMARY OF RESULTS

Characterization of the tumor microenvironment (paper I)

Tumor cell hypoxia and proliferation was analyzed in relation to the tumor vasculature. Fourteen tumors from 11 first generation xenograft lines of human head and neck squamous cell carcinomas were studied.

Hypoxia, detected with pimonidazole, was found in all tumors. The tumors were divided into three groups: patchy, mixed, and ribbon-like, based on qualitative morphological features. The hypoxic patterns were quantified according to an elongation index (EIH, length/width) that ranged from 2.0 to 28.3 and showed a good correlation with the qualitative analysis. The bands of hypoxia in the ribbon-like tumors often seemed to be in close relation to networks of vessels arranged in sheets (lower panels of Figure 1, paper I). These networks were not found in the patchy tumors. Analysis of the distribution of hypoxia throughout the tumor section or as function of distance to the vasculature did not reveal any differences between tumors with different hypoxic patterns.

Analysis of proliferation (BrdUrd labeling) did not reveal any distinguishable proliferation patterns, neither in relation to the hypoxic patterns nor in relation to the distance to the vasculature.

Analysis of EIH showed a significant correlation with the hypoxic fraction (HF) but not with other measured parameters. However, there was a trend towards a higher labeling index and a larger fraction of necrosis with increased EIH.

Analysis of the hypoxic fraction as function of distance to the vasculature showed that in all tumor lines varying amounts of hypoxia was present already within the first 50 µm from the vasculature, indicating the presence of perfusion limited (acute) hypoxia. In most tumors the HF increased with the distance to the vasculature. In addition, the labeling index of BrdUrd was generally highest adjacent to the vasculature with a subsequent decrease at increasing distance. Although these parameters were analyzed on consecutive tissue sections the results indicated co-existence of hypoxic and proliferating cells that was most pronounced at 100-150 µm from the vasculature (Figure 5 and 8 paper I).
The lifespan of hypoxic cells in three different tumor lines (paper II)

A double hypoxic marker assay was applied to determine the lifespan of the hypoxic tumor cell population. Three tumor lines were studied, SCCNi3 (patchy hypoxic pattern), and the MEC82 and C38 (ribbon like hypoxic patterns). The SCCNi3 and MEC82 are derived from human head and neck tumors of squamous and mucoepidermoid origin, respectively. C38 is a murine colon tumor line transplanted in C75BL/6 black mice.

Pimonidazole was always injected first, followed by CCI-103F at varying time intervals after pimonidazole, but always 2.5 h before tumor harvest. There was a good spatial match between the two markers at short injection intervals, but pimonidazole always covered a larger surface fraction than pimonidazole in the binary images (Figure 3 paper II). Therefore, the ratio for the mean hypoxic fractions of the first hypoxic marker pimonidazole (HF1) and the second hypoxic marker CCI-103F (HF2) was set to 1.0 for the control tumors of each tumor line. The ratios for the subsequent injection intervals were normalized relative to the control values, and from the resulting curves the half-life of the hypoxic cells was calculated for each line. The half-life (95% confidence interval) of the hypoxic cells was 49h (28-185h) for SCCNi3, 23h (16-41h) for MEC82 and 17h (15-19h) for C38 (Figure 4 paper III). The ranking of the potential volume doubling times in these tumor lines was related to the ranking of the hypoxic lifespan.

The hypoxic markers are only reduced and bound in viable hypoxic tumor cells, and are not reduced in necrotic tumor areas. Once reduced and bound to intracellular proteins the, hypoxic marker adducts are stable in the tissue. Therefore label from dying cells was expected in necrotic areas at longer time intervals. Qualitative analysis of the tumor sections showed that the rapid disappearance of pimonidazole labeled hypoxic cells in C38 and MEC82 indeed corresponded with cell debris staining positive for pimonidazole in the necrotic areas. This was already evident at the 16h injection interval in the C38 line and at the 24h injection interval in the MEC82 line.

Dynamic measurements of hypoxia after oxygenation modifying treatment (paper III)

The experiments were done with the human laryngeal squamous cell carcinoma xenograft line SCCNi3. A double bioreductive hypoxic cell marker assay was developed to study changes in tumor hypoxia after tumor oxygenation modifying treatments. The two hypoxic markers, pimonidazole and CCI-103F, were sequentially...
injected in each tumor. The oxygen modifying treatment; carbogen breathing (95% O₂ and 5% CO₂) or hydralazine was administered between the hypoxic marker injections.

A good spatial match was seen between the hypoxic markers in non-treated animals (Figure 1A and 2A paper III). Carbogen caused a drastic and rapid reduction of the mean hypoxic fraction. It decreased from 7% detected by CCI-103F before treatment to 3% detected by pimonidazole administered during carbogen breathing \( p=0.009 \), (Figure 4 paper III). The decrease of hypoxia did not occur randomly, and was most pronounced close to well-perfused regions where hypoxia often completely disappeared.

Hydralazine caused vasodilatation of the vasculature in the surrounding normal tissue. This resulted in a reduction of the tumor perfusion, and subsequently in a significant increase of the mean hypoxic fraction from 16% to 40% \( \text{ps}0.05 \).

Thus with consecutive injections of two bio-reductive markers changes in tumor hypoxia can be quantified in relation to the (perfused) tumor vasculature.

**Dynamic changes in hypoxia after treatment with the hypoxic cytotoxin hydralazine (IV)**

The effect of the hypoxic cytotoxin tirapazamine (TPZ) on the hypoxic tumor cell population was studied with the double hypoxic marker method described in paper III. TPZ was injected between the subsequent hypoxic marker injections. The dynamic changes in hypoxia were analyzed with a triple staining of the endothelium plus the two hypoxic markers within one tumor section. Therefore analysis could be done on a cell-by-cell basis. Qualitative and quantitative analysis showed that TPZ induced a decrease of the HF reaching a nadir about 3.5h after TPZ injection followed by rehypoxification 24h after treatment (Figure 1 and 2 paper IV). Qualitative analysis shows that the effect of TPZ is largest at intermediate distances from the tumor vasculature. In addition the triple staining showed that the *same cells* that were hypoxic before TPZ injection had become hypoxic again 24h after TPZ treatment.

A triple staining of vasculature, caspase 3 and CCI-103F showed a trend towards increased apoptosis in the hypoxic cell compartment at 24h and 48 after TPZ administration.
Tumor microenvironment and model systems

The main focus of this thesis was to study the dynamics of hypoxic tumor cells in relation to the vascular network in an experimental setting. These were mainly performed in human head and neck tumors grown as xenografts. Xenografts consist of tumor cells of the donor genotype (for example human), while the vascular and stroma components are of host origin (for example mouse). This was shown by comparison of binding patterns of anti-human and anti-mouse antibodies in xenografted human head and neck tumors (Warenius, 1980).

The use of xenografts allows *in vivo* analysis of the interplay between different tumor cell populations in solid tumors. Clinically relevant treatment strategies can be evaluated with respect to the tumor microenvironment, using endpoints such as growth delay. Most human xenograft lines only survive *in vivo*, and must be passaged from animal to animal. The advantage of xenografts is that these tumor lines are the best experimental model systems for human tumors. A disadvantage is however the high cost and the large number of animal needed for maintenance of the tumor lines. A number of well-known tumor lines are further transformed and can be grown in cell culture, as spheroids and as xenografts, such as the SiHa and WiDr xenografts (Durand & Sham, 1998). An advantage of these tumor lines is that they can be used both *in vivo* and *in vitro*. A disadvantage is that these tumor models are further transformed and could have lost some of the original properties that may affect the tumor microenvironment.

Comparisons of several human head and neck tumors grown as xenografts (paper I) showed that the human tumor cells induced specific growth patterns of the tumor vasculature. It was evident that these vascular characteristics were tumor specific since the vascular architecture differed between xenograft lines, and remained constant when passaged. This is in agreement with other studies of human xenografts. Examples of the donor dependence of the vascular architecture have been reported for human glioma and melanoma xenograft lines in nude mice (Bernsen et al., 1995; Solesvik et al., 1982). In addition, vascular corrosion casting studies showed that the three-dimensional architectures of tumor vessels were tumor line dependent (Konerding et al., 1999).

In the present study the hypoxic patterns identified by morphological classification were tumor line dependent and were retained over subsequent passages in the individual tumor lines (paper I). The hypoxic patterns seemed to be related to the...
vascular architecture, These were similar to the hypoxic patterns found in primary human head and neck tumors (Wijffels et al., 2000). It can be concluded that these xenograft lines can be used as models for the microenvironment of human head and neck tumors. The hypoxic patterns were also similar to hypoxic patterns reported for human and canine tumors elsewhere (Cline et al., 1994; Kennedy et al., 1997).

Double hypoxic marker assay

To study the dynamics of hypoxic tumor cells in relation to the tissue architecture a double hypoxic marker assay was developed. This was first presented in paper III. The two hypoxic markers pimonidazole and CCI-103F were consecutively injected with oxygenation modifying treatment in between, revealing both increased and decreased levels of hypoxia. Moreover this assay was used to quantify hypoxic cell half-lives (paper II) and the effect of treatment with the hypoxic cytotoxin tirapazamine on tissue oxygenation (paper IV).

Pimonidazole accumulates more rapid in hypoxic areas than CCI-103F. In addition reduced and bound pimonidazole adducts have been shown to be more stable than CCI-103F adducts in canine tumors (Azuma et al., 1997). Therefore the injection sequence of the two hypoxic markers varied depending on the length of the experiments.

Binding profiles of the two hypoxic markers

Measurements of intensity profiles in paper II of the two consecutively injected hypoxic markers, pimonidazole and CCI-103F, resulted in hypoxic profiles with a similar shape but different absolute intensities. This indicates that metabolism and binding of the two markers occurred in the same cells, but that the absolute staining intensities differed. Pimonidazole and CCI-103F differ in the side chain that affects the hydrophilicity and intracellular accumulation of the markers. This affects the staining intensities of the two markers as well as primary antibody affinities, dilutions and incubation times with the antibodies. This is supported by the fact that the binding patterns of pimonidazole and CCI-103F are similar to that for misonidazole (Arteel et al., 1995; Raleigh et al., 1987). Misonidazole shows increased binding at and below 10 mm Hg (Gross et al., 1995) which indicates similar oxygen dependence for the pimonidazole and CCI-103F.
Another clinically applicable 2-nitroimidazole is EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide]. Based on the biochemical properties EF5 is expected to have similar oxygen dependence as pimonidazole and CCI-103F, which would result in similar binding profiles of hypoxic cells labeled with any of the three markers. EF5 has another unique side chain that can be detected with antibodies (Evans et al., 1995) and is easier to dissolve than CCI-103F. Therefore EF5 is expected to be a good candidate for use in the double hypoxic marker assay.

Spatial match/mismatch of the hypoxic markers

One would expect from the similar intensity profiles of pimonidazole and CCI-103F that the hypoxic fractions estimated by the two hypoxic markers would be identical when injected concurrently or with short intervals. However this was not the case. The ratios of the hypoxic fractions for pimonidazole over CCI-103F were always larger than one. One reason is the difference in signal to noise ratio, which is lower for CCI-103F than for pimonidazole. This might be caused by larger variations of the background levels of CCI-103F than of pimonidazole. These differences affect the threshold value and the resulting hypoxic fraction calculated from the binary images. A universal correction factor for the hypoxic fraction of CCI-103F could not be established due to the larger intratumoral differences observed for CCI-103F than for pimonidazole binding.

Delivery problems of CCI-103F compared to pimonidazole could be ruled out since intensity measurements showed similar intensity profiles for both markers. In addition, differences in spatial match between the markers were seen at intermediate distances from the vasculature, while at further distances the spatial match for the two markers was better (Figure 3 and 5 paper II). Moreover comparison of the untreated control tumors of the hypoxic life-span study (paper II) and the tirapazamine study (paper IV) showed that the ratio between the two markers was not affected by the injection sequence.

Therefore it was concluded that differences in signal to noise ratio of the two hypoxic markers and a difference in staining intensity were the main reasons for the small differences in hypoxic fractions for pimonidazole and CCI-103F at short injection intervals. However, an excellent overall spatial match of these two markers still was found.
Detection of perfusion limited (acute) hypoxia

Two forms of hypoxia have been shown to exist in solid tumors; diffusion-limited chronic hypoxia (Tannock, 1968; Thomlinson & Gray, 1955) and transient perfusion-limited acute hypoxia (Brown, 1979). These forms of hypoxia represent the most extreme forms of hypoxia, and intermediates between these two forms are most likely to be present in the tumor tissue. It was demonstrated that longitudinal gradients in pO₂ exist within tumor vessels, which could be related to the tissue pO₂ (Dewhirst et al., 1999). In addition, it has been shown that small fluctuations in the blood perfusion are more common than complete vascular closures or openings (Durand, 2001).

The binding of the bioreductive hypoxic markers depends on the absolute levels of pO₂ and the total distribution of hypoxia. The immunohistochemical staining patterns of these markers correspond with the expected areas of diffusion-limited hypoxia. The presence of hypoxia within the first 50 µm from tumor vessels, which was found in the characterization study (paper I), provides circumstantial evidence for detection of perfusion-limited hypoxia with bio-reductive hypoxic markers. However, it could be argued that these short distances to hypoxia would be caused by a very low pO₂ in the tumor vessels or of a complete vascular shutdown of long duration.

In the hypoxic life-span study (paper II) evidence for detection of perfusion limited hypoxia with hypoxic markers was found at the 2h injection interval in the MEC82 line (Figure 2 paper II). A mismatch, with increased staining of the second hypoxic marker, CCI-103F, relative to the first hypoxic marker, pimonidazole, was found (see arrows). Measurement of hypoxic marker intensity profiles (unpublished data) showed large spatial variations of the CCI-103F staining profiles relative to the pimonidazole staining profiles in areas with perfusion-limited hypoxia.

In addition, a gradient of CCI-103F staining intensities along the feeding vessel (Figure 2, paper II, FV) from the tumor periphery to the area with transient perfusion limited hypoxia (Figure 2, paper II, AH) was indicative for a decreasing oxygen gradient along the vessel. This indicates that temporal fluctuations of the perfusion rate in MEC 82 have resulted in regions with transient hypoxia in this specific tumor. A closer examination of all tumors in the MEC82 line and in the other two tumor lines (C38 and SCCNij3) has indicated that these fluctuations of hypoxia occur at varying degrees in all three lines but that this was most pronounced in MEC82.
DISCUSSION

Hypoxic patterns

The hypoxic patterns describe the global organization of hypoxic cells in relation to the vascular architecture. A corded structure of the hypoxic cell organization in relation to hypoxia with either peripheral stroma, followed by hypoxia and central necrosis (Thomlinson & Gray, 1955) or a central blood vessel surrounded by hypoxic cells at a distance followed by necrosis (Tannock, 1968) has been described. Thus both models were associated with diffusion-limited hypoxia. Generally only experimental tumors have been described as being truly corded. In the xenograft lines with a patchy structure of hypoxia, the tumor cells were organized as clusters of grapes around the vessels, while in the xenograft lines with a ribbon-like hypoxic structure the tumor cells were lined up as bands along the tumor vasculature (Figure 1 paper 1).

The patchy hypoxic pattern could be explained as several tumor cords with a central blood vessel and at a distance, diffusion-limited hypoxia of the type described by (Tannock, 1968). Between the tumor cords corners of tissue could be present at inter-vascular distances exceeding the oxygen diffusion distance, resulting in “spots” of diffusion-limited hypoxia with necrosis at further distances from the vessels in larger hypoxic areas.

The ribbon-like hypoxia pattern is characterized of hypoxic cells located along elongated networks of vessels with necrosis at further distances. This organization corresponds with the description of Tomlinson and Gray with peripheral stroma and central necrosis (Thomlinson & Gray, 1955). Diffusion-limited hypoxia would arise beyond the diffusion distance of oxygen, followed by central necrosis. A trend towards higher hypoxic fractions, and BrdUrd labeling indices together with a higher proportion of non-viable tissue in the ribbon-like tumor lines indicated a higher cell turnover in these tumors than in tumor lines with a patchy hypoxic pattern.

Hypoxic lifespan and its relation to hypoxic patterns

Hypoxia inhibits cell proliferation and eventually leads to cell death, but hypoxia also induces signals for angiogenesis and may lead to a more aggressive phenotype. The double hypoxic marker assay (paper II) showed that hypoxic cells in the two ribbon-like tumor lines, MEC82 and C38, had a higher turnover rate than in the patchy tumor line, SCCNij3. These results were consistent with the trend towards a higher labeling index and a larger proportion of non-viable tissue for the ribbon-like xenograft lines that described in paper I.
Generally murine tumor lines tend to have faster growth kinetics than human xenograft lines, and it cannot be excluded that this plays a role in the rapid kinetics of the C38 line. However, differences between the labeling indices and T-pot values for the ribbon-like murine C38 line and human xenografted MEC82 line were smaller than the differences between the two xenograft lines with different hypoxic patterns (MEC82 and SCCNij3). This suggests that the hypoxic patterns could be related to the dynamics of the hypoxic cell population. Tumor lines with a ribbon-like hypoxic pattern might have a shorter half-life of hypoxia, indicating that these cells have a limited ability to adapt to the hypoxic environment. Tumor lines with patchy hypoxic patterns tend to have a lower amount of necrosis and a lower labeling index. These features may be extrapolated to the data for the SCCNij3 line, and may reflect acquired hypoxic tolerance.

Hypoxic tolerance requires the ability of hypoxic cells to reduce the energy demand and translation resulting in an altered proteome. It has been suggested that tumors with hypoxic tolerance e.g. prolonged survival at low oxygen tensions could play a role in tumor growth by providing a continuous angiogenic signal (Wouters et al., 2003). This has been supported by recent observations, showing that the tumor microenvironment affects the half-life of hypoxic cells. The lifespan of hypoxic cells of the SiHa line was 3-5 days in spheroids and 4-10 in xenografts (Durand & Sham, 1998). Furthermore, the extra-cellular pH has been shown to affect the viability and clonogenicity of hypoxic cells (Schmaltz et al., 1998), and cell-sorting experiments indicated that selection of tumor cell subpopulations with a reduced vascular dependence might occur during disease progression (Yu et al., 2001). Selection of sub-lines with low vascular dependence resulted in higher take rates and a more rapid growth of a melanoma xenograft line in nude mice (Yu et al., 2001).

**Hypoxia and anti-cancer treatments**

Tumor cell hypoxia reduces the radiosensitivity, and three general treatment strategies have been designed to overcome hypoxic treatment resistance during radiotherapy. These are: radiosensitizers such as misonidazole, oxygen-modifying treatments where carbogen breathing is applied and selective hypoxic cytotoxins such as tirapazamine. A combination of tirapazamine with irradiation could result in complementary cell kill (Brown & Siim, 1996; Wouters et al., 2002).

By using the newly developed double hypoxic marker method we were able to study the dynamics of the hypoxic cell population after oxygenation modifying
treatment (paper III) and after treatment with the hypoxic cytotoxin tirapazamine, (paper IV).

The SCCNij3 line was treated with the high oxygen content gas carbogen (95% O₂ and 5% CO₂), and the effect of carbogen was compared with pretreatment levels in the individual tumors (paper III). The effect of carbogen was most pronounced in the vicinity of perfused vessels, indicating that the intermediate hypoxic cell might benefit most from the increased oxygenation. Also increased levels of hypoxia could be measured after treatment with the vasodilator hydralazine, which divert the blood flow from the tumor vasculature to the surrounding normal vasculature.

Treatment with tirapazamine in the SCCNij3 line (paper IV) was most efficient at changing the oxygenation at intermediate distances from the vasculature. Tirapazamine is a highly diffusible molecule that is most effective at very low pO₂ levels (Brown, 1993), but it was shown that tirapazamine might be fully reduced before it reaches the most distant hypoxic cells (Kyle & Minchinton, 1999). Repeated administrations are feasible in mice (Spiegel et al., 1993) and increased cell kill is expected upon repeated administrations.

Tirapazamine treatment caused a transient decrease in tumor hypoxia that reached a nadir approximately 3.5h after administration. Subsequently, tumor hypoxia slowly increased, and was almost back to pre-treatment levels at 48h after tirapazamine injection. The kinetics of reoxygenation and rehypoxification in the SCCNij3 after tirapazamine (paper IV) was similar to the kinetics observed in this line after irradiation (Bussink et al., 2000a). After tirapazamine treatment a similar pattern of reoxygenation followed by rehypoxification was reported for the SCCVII line both for tirapazamine and irradiation (Kim & Brown, 1994). The kinetics differed between SCCNij3 in this study and the SCCVII line. When the nadir for SCCNij3 was reached already pretreatment levels were seen in the SCCVII line. Although methodological differences cannot be excluded, this suggests a tumor line dependence of the tirapazamine response that might be related to the dynamics of hypoxic cells.

Reproducibility of results: impact of different image analysis systems and staining protocols

The larger hypoxic fraction for pimonidazole compared to CCI-103F was consistent both for experiments analyzed on the 8-bit and 12-bit image analysis systems. A comparison of the results obtained for the SCCNij3 line with the two image
DISCUSSION

Analysis systems was made for control tumors in two different double hypoxic marker studies (paper II and III).

The staining protocols and detection of the markers were optimized for the system used in each experiment. For the 8-bit system the two markers were stained on consecutive tissue sections, but were detected with the same fluorochrome. For the 12-bit system, a triple staining for both hypoxic markers and vasculature was done, using different fluorochromes for the hypoxic markers (green for pimonidazole and red for CCI-103F) to achieve an exact spatial analysis of the distribution of the two hypoxic markers. In addition the image processing steps and the resolution were different in the two studies. The same definition of the hypoxic fraction (the tumor surface stained for the hypoxic marker relative to the total tumor surface) was used for the two different image analysis systems. Although the differences between the hypoxic fractions in the two experimental settings were small, the differences were larger for CCI-103F than for pimonidazole. Thus, it can be concluded that pimonidazole is less sensitive to variations in staining protocols and experimental settings than CCI-103F.

The results in this study show that although the differences are not dramatic, different experimental settings affect the hypoxic fractions. In addition there are several different definitions of hypoxic fractions in the literature further complicating comparisons of absolute values between different studies. Therefore comparisons of absolute values must be done with caution, while comparisons of relative differences such as the ranking of hypoxic fractions or relative differences between control tumors and treated tumors between the different systems are less problematic.

Future Aims

The ultimate goal of ongoing research is to achieve individualized treatments based on the vascular architecture and microenvironmental parameters. The results presented in this thesis is one step forward towards understanding the role of the dynamics of the tumor microenvironment in the response to anti-cancer treatments.

In the studies in paper II and paper IV the proliferation marker BrdUrd was injected together with pimonidazole, and the proliferation marker IdUrd was injected together with CCI-103F. A triple staining protocol of hypoxia, proliferation and vasculature, as well as a triple staining of vasculature, BrdUrd, and IdUrd are currently under development. The relation between hypoxia and proliferation will be analyzed in non-treated tumors. Furthermore the double hypoxic marker method will be used to
analyze the effects of cytotoxic drugs on the balance between proliferating and hypoxic subpopulations.

The double hypoxic marker method is a robust tool that can be used to design and test the efficiency and toxicity of clinically relevant complementary treatment strategies, and will be used to further explore the relationships between the dynamics of the tumor microenvironment and the effect of different treatment modalities.
CONCLUSIONS

The tumor microenvironment is characterized by an aberrant and dysfunctional vascular supply that may lead to hypoxic regions. Generally a gradient of oxygen tensions is present in the tumor tissue, with relatively oxic proliferating cells near the vasculature. At increasing distances the level of tissue hypoxia increases, with a corresponding decrease of the proliferation rate. Beyond a critical threshold in the oxygen and nutritional gradients necrotic cells are found.

In this study the tumor microenvironment was analyzed with a multi parameter method including the vasculature, proliferating BrdUrd-labeled cells and hypoxic cells labeled with pimonidazole or CCI-103F. A double hypoxic marker assay was developed to study the dynamics of the hypoxic cell population in experimental tumor models.

From the papers included in this thesis the following conclusions can be drawn:

I. Characterization of 11 human head and neck cancer xenografts revealed two main hypoxic patterns: patchy and ribbon-like. These seem to be correlated with the vascular architecture. However, no difference of hypoxia as a function of distance to nearest blood vessel was found. Moreover, the hypoxic patterns were not related to tumor differentiation grade. There was a trend towards a higher hypoxic fraction, and BrdUrd labeling index in the ribbon-like tumors. In addition, there was a larger amount of necrosis in these tumors. Thus the hypoxic pattern may reflect the dynamics of the tumor microenvironment.

II. The double hypoxic marker method developed in this study was used to study the half-life of hypoxic tumor cells. These were labeled with pimonidazole at different time intervals and with CCI-103F 2.5 h before harvest. The ratios between the hypoxic fractions of these two markers were used to determine the half-lives of the hypoxic cells in three different tumor lines. The half-life of the hypoxic tumor lines cells ranged from 17 h to 49 h. The ranking of the hypoxic half-lives corresponded with the ranking of the potential tumor doubling time, but not with the actual tumor volume doubling times.

III. The double hypoxic marker assay was used to study changes caused by oxygenation modifying treatments in the SCCNij3 line. Carbogen (95% O₂ and
5%CO₂) caused a decrease of hypoxia, which was most pronounced at intermediate distances from tumor vessels. Hydralazine, a vasodilator that diverts the blood flow from the tumor vasculature to the surrounding normal tissue was used to show that both increased and decreased levels of hypoxia could be measured with this assay.

IV. The double hypoxic marker method was used to study the effect of the hypoxic cytotoxin tirapazamine on the hypoxic tumor cell population. A decrease in the hypoxic fraction was seen shortly after tirapazamine injection. The nadir was reached between 1-3.5 h after tirapazamine administration. The reoxygenation was followed by a rehypoxification, and at 48h after tirapazamine injection the hypoxic fractions were almost back to control levels.

The three studies with the double hypoxic marker assay have shown this to be a robust method. It could be used to measure both dynamic changes of the hypoxic cell population in non-treated tumors, as well as the effects of oxygen modifying treatments and the effect of cytotoxic drugs on hypoxic cell populations with high spatial resolution relative to the tissue architecture.
This work would not have been accomplished without the help and support from colleagues, friends, and family. I would like to use this opportunity to thank you all for the help and I would like to emphasize a few of you here.

I especially want to thank my supervisor professor Bert van der Kogel for the advice, constructive criticism during this project and for letting me work in Nijmegen and professor Anders Bergh for the support. I want to thank professor Julie Denekamp (in memorandum) who first encouraged me to study the field of radiobiology. Her contribution through discussions and advice has been invaluable for me.

This work was performed both at the Departments of Radiation Sciences, Umeå, Clinical Bioemical Research the unit for Pathology Umeå and at the Department of Radiation Oncology, University Medical Center Nijmegen, The Netherlands. I would like to thank all of you that have been my colleges during this work. I especially want to mention some of you.

Jan Bussink, for the feedback on experimental designs and critical reading of the manuscripts.

Paul Rijken for stimulating discussions and the continuous development of the image analysis systems, and for being a supportive roommate during the years in Nijmegen.

Hans Kaanders for providing the primary material for establishment of the xenograft lines and stimulating co-autorship.

Hans Peters, for development of immunohistochemical staining protocols. Jasper Lok and Wenny Peeters, for all help with stainings and scanning.

Marielle Phillipens for all the support.

Geert Poelen, Bianca Lemmers and Debbie Smits at the Central Animal Laboratory, Nijmegen for excellent animal care.

Professor Jim Raleigh and professor Adrian Begg, for co-authorship and stimulating collaborations.
Professor emeritus Bo Littbrand for giving me the opportunity to do this work and for the support.

Among the colleagues in Umeå, I especially want to mention Christina Bergström, Iuliana and Alexandru Dasu, and Irina Goritskaya.

Katrin Sundh, Cecilia Elofsson, and Anna Wernblom provided excellent administrative service.

All my friends in Sweden, the Netherlands, and the rest of the world for the support during my scientific and personal ups and downs. Ylva Hedberg, Christer Mehle, Maria Nikolova and Charlotte Thysell though far away always close to me. Mariska Drenth for the support and introduction to the Netherlands. Chris Gerrist for encouragement and consideration.

Last but not the least I want to thank my family. Especially my mother Inger for all support and long phone calls, my father Stig, and my brother Peter, you are always there for me when I need you. Without the stable basis and all support you have given me I would not have made it.

Funding was kindly provided from the Cancer Research Foundation in Northern Sweden, the Sir Samuel Scott of Yews Trust, the United Kingdom, and the Dutch Cancer Society.
References


REFERENCES


REFERENCES


REFERENCES


References


REFERENCES


REFERENCES


-72-


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


