Effects of treatments with angiogenesis inhibitors on tumor stroma in animal experimental models of child cancer Neuroblastoma

Mahamed Shiikh Dahir

Degree Project in Toxicology, 30 hp, Spring semester 2013

Supervisor: Faranak Azarbayjani
Examiner: Eva Brittebo

Division for toxicology
Department of Pharmaceutical Biosciences
Faculty of Pharmacy
Uppsala University
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Abstract

Neuroblastoma, a neuroendocrine tumor, is the most common cancer in infancy. 75% of those affected are under the age of 5. The disease is heterogeneous and survival rate is low.

Current treatment of neuroblastoma consists of surgery, radiation and chemotherapy, where the targets for the treatment are the malign cells. Due to the cancer cells instable genome there is a risk for resistance development. This negatively impacts the treatments goal of hindering tumor growth and spread. Tumor growth is not only determined by malign cells but also the interactions of those tumor cells with tumor vessels and different types of cells in the tumor stroma.

The aim of this paper is to develop a relevant histological method to study the properties of tumor stroma in tumor sections retrieved from human NB tumor xenografts in mice treated with angiogenesis inhibitors SU11657 and Zoledronic acid. The study is a continuation of previous studies with the inhibitors which have shown good effect on tumor growth and angiogenesis on neuroblastoma.

In the short term treatment with SU11657 and Zoledron acid showed that tumor growth declined. In the longer treatment with SU11657 the growth didn’t decline with the same rate compared to the short term treatment. Angiogenesis on the other hand decreased in all the treatments independent of treatment duration. The histological staining with Sirius red revealed that treated tumors had an increased amount of stroma compared to the untreated tumors.

In conclusion the relative increase of tumor volume, decreased number of vessels and expansion of tumor stroma in the longer treatment with SU11657 indicated that tumors might survive the angiogenesis inhibitor treatment through expansion/activation of its stroma. The histological staining with Sirius red in saturated picric acid marked the collagen, i.e. stroma, well and enabled quantification of the stroma.
1. Introduction

1.1. Childhood cancer and Neuroblastoma

The years of studying cancer has revealed the complexity of the disease and its involvement in the dynamic change of the genome. The foundation for a cell undergoing cancer transformation has been determined to come through the following 6 modifications (the hallmarks of cancer): self-sufficiency in growth signals, evading apoptosis, insensitivity to anti-growth signals, tissue invasion & metastasis, limitless replicative potential and sustained angiogenesis (Hanahan & Weinberg, 2000).

The 5-year survival rate for children with cancer has improved dramatically these last 50 years and in most developed countries the survival rate exceeds 70 %. The mortality rate of certain other types of cancer such as neuroblastoma however remains relatively high beyond the 5-year monitoring period (Johnston et al, 2010).

Neuroblastoma is a malignant tumor, the most common malignant disease in children, 75 % of the children diagnosed with the disease being under 5 years-old. The disease is considerably heterogeneous and the survival rate is still poor. The survival rate is dependent on the age at diagnosis, tumor stage and certain biological markers. Current treatments of neuroblastoma are mainly: surgery, radiation and chemotherapy. The negative effects of the treatments are resistance to treatment, risk for growth retardation, early onset of cardiac failure, infertility and secondary malignancies. Considering these adverse effects, there is a need for new treatment strategies. One of these approaches is the inhibition of tumor angiogenesis (Bäckman et al, 2002).

1.2. Causes and prognosis of neuroblastoma

The tumors in neuroblastoma are derived from neural crest cells and commonly arise from the adrenal gland or paraspinal sympathetic ganglia. The causes for NB remain still unknown. No environmental or parental exposures that significantly impacts disease occurrences have yet been identified. Usually the cancer occurs spontaneously
and sporadically but in 1%-2% of cases there is a family history. The familial NB hints at an underlying genetic abnormality, though studies have failed to identify a specific tumor suppressor gene that is responsible for NB (Weinstein et al, 2003).

Neuroblastoma is noteworthy for its broad spectrum of clinical behavior. As mentioned earlier prognosis varies with genetic abnormalities, age at diagnosis, tumor stage (see table 1, INSS) and treatment for NB is based on surgery, chemotherapy and radiotherapy. The treatment is assigned through the prognostic categories of INSS.

Some of the NB tumors regress spontaneously (occurs most often in stage 4S) or differentiate to benign ganglioneuromas. Most children older than 1 year with the advanced-stage of NB die despite intensive multimodality therapy. In contrast children less than one year of age and with stages 1 and 2 can be cured with surgery alone (Weinstein et al, 2003).

Table 1. Staging system for neuroblastoma according to the international neuroblastoma staging system (INSS) (Pizzo and Poplack, 1997).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Localized tumor confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 2A</td>
<td>Unilateral tumor with incomplete gross excision; identifiable ipsilateral and contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 2B</td>
<td>Unilateral tumor with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; identifiable contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Tumor infiltrating across the midline with or without regional lymph node involvement; or unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral lymph node involvement.</td>
</tr>
</tbody>
</table>
Stage 4  Dissemination of tumor to distant lymph nodes, bone, bone marrow, liver, or to other organs.

Stage 4S  Stage 1 or 2 otherwise in children aged < 1 year, with metastases in liver, skin, or bone marrow but not in the bone.

1.3. Angiogenic inhibitors and chemotherapy

Chemotherapy causes toxicities such as bone marrow depression and enteritis. Another problem is acquired drug resistance since chemotherapy targets tumor cells and other proliferating cells, which are genetically unstable and prone to mutations. In contrast angiogenic inhibitors targets activated endothelial cells and are less likely to have the same problems, such as development of resistance, since these cells are untransformed and have a stable genome (Rosen, 2000).

The inhibitors prevent angiogenesis which is the process by which capillaries sprout from pre-existing blood vessels. The process involves several factors, so called angiogenic factors, which acts both stimulating and inhibiting. Pro-angiogenic factors are upregulated under physiological conditions, external stimuli such as hypoxia, hypoglycemia and mechanical stress which activate angiogenesis. Otherwise the factors are maintained in a dormant state. Angiogenesis is characterized by processes such as increased vascular permeability, dissolution of vascular basal membranes, extracellular matrix degradation, endothelial cell proliferation and migration. Tumor angiogenesis differs from normal physiological angiogenesis. Tumors interact with stroma and angiogenesis. Stroma is the connective tissue, framework, inside biological tissue, cell or organ. Tumors growth is influenced by both angiogenesis and stroma. Tumor angiogenesis forms new vessels constantly and the tumor vessels are unstable. The vessels are structurally and functionally abnormal and because of these abnormalities distinction between normal and tumor vessel is possible (Fox et al, 2001)
Angiogenic inhibitors are expected to be cytostatic and because of the different cellular targets, angiogenic combined with standard chemotherapy may be useful against tumors (Rosen, 2000).

1.4. In vivo models

Since the NB incidence is comparatively low this limits the number of clinical trials that can be run simultaneously thus limiting new treatment strategies. There is therefore a need for clinically relevant, reliable and reproducible animal models.

Subcutaneous (s.c.) models are animal models where tumor cells are injected under the skin of immunodeficient mice. This method is preferred for being fast, reliable and easy way to assess tumor growth, treatment efficacy and angiogenesis. S.c. tumors are easier to measure daily due to their accessibility (Fuchs, 2009).

2. Purpose

The aim of this paper is to develop a relevant histological method to study the properties of tumor stroma in tumor sections retrieved from human NB tumor xenografts in mice treated with angiogenic inhibitors. This is a continuation from previous studies where tumors have been developed in animal experimental models with human neuroblastoma cells injected subcutaneous in immune defective mice. These tumor-carrying animals have been treated with angiogenic inhibitors, SU11657 and Zoledronic Acid. The effect of the treatments on tumor growth and stroma has partly been studied by quantifying the vessel number and morphology in earlier studies. This study is conducted to further illuminate the characteristics of the tumor stroma with focus on formation of collagen in treated compared to untreated stroma.
3. Methods

3.1. Previous studies

Animal studies
The experiments were approved by the regional ethics committee for animal research. For SU11657 some animals were treated for 20 days while others continued the treatments. (Bäckman & Christofferson, 2005; Bäckman et al, 2008)

Xenografting
In previous studies (Bäckman, 2003), neuroblastoma cell line (IMR-32) was used for xenografting in NMRI nu/nu mice (B & M, Ry, Denmark*).

The mice are born lacking CD4+, CD8+ T cell activity but they can however develop cellular immunity with maturation. Therefore the mice were xenografted with tumor cells (30x10⁶ cells) at the age of 7-8 weeks (body weight 25-30 g males, 20-25 g females).

When the tumors were palpable (~0.1 ml) measurements for tumor volume began. At the volume of ~300 mm³ the treatment began after animal randomization. The mice received treatment within 7-10 weeks (IMR-32) after xenotransplantation. Vehicle were given to the control animals, SU11657 was given orally daily (40 mg/kg/day) and Zoledronic acid (100µg/kg) was given s.c. daily (Bäckman & Christofferson, 2005; Bäckman et al, 2008)

3.2. Perfusion fixation and autopsy

When the tumor burden in control animals reached a maximum volume of 4-5 ml, the animals were anesthetized and perfusion fixed through the thoracic aorta, with 4% paraformaldehyde (Millonig’s buffer, pH 7.4, 37°C). The tumors were excised and their weights were recorded. The tumors were then immersion-fixed in 4% formaldehyde, a week before dehydration and paraffin embedding (Bäckman & Christofferson, 2005; Bäckman et al, 2008).
3.3. Current study: Sectioning and Staining

The paraffin embedded tumors were sliced at 3 µm and put on 3-aminopropyltriethoxysilane-treated glass slides (Sigma). To study the extent of tumor stroma in these tumors, representative tumors from the control and treated animals were chosen. These slices were stained using three staining technique, Sirius red in saturated picric acid, Hematoxylin & Eosin and Picro-Mallory.

Hematoxylin & Eosin (H&E)
The sections were deparaffinated with xylen, rehydrated in graded ethanol series and washed in tap water. The sections were stained in Mayers Hematoxylin & Eosin 0,2 % for 10 minutes. After staining the samples, staining intensities were adjusted by washing in tap water and 95 % ethanol. The samples were then dehydrated in graded ethanol series and xylene, mounted with coverslips.

Picro-Mallory
Wash sections in distilled water, stain in Celestine blue, 3 minutes. Wash in water then stain in Carazzi Heamalum 3 minutes and then wash in water. Blue in Scotts tap water, wash in water and rinse in 95 % ethanol. Stain in picro-orange 2 minutes, wash in water until red blood cells are yellow, stain in acid fuchsin 5 minutes. Wash in water, stain in equal parts Picro-orange and 80 % ethanol. Differentiate in phosphomolybdic acid for 8 minutes, wash in water and stain in analine blue, 8 minutes. Wash in water and dehydrate. Mount sections in DPX. The staining with picro-mallory was automated and done by Rättsmedicinalverket.

Sirius red in saturated picric acid
The sections were deparaffinated with xylen, rehydrated in graded ethanol series and destilles water. The sections were then stained with Sirius red in saturated picric acid for 20 minutes. After staining the samples staining intensities were adjusted by blotting.
with filter paper. The samples were then dehydrated in 99 % ethanol series and xylene, mounted with coverslips.

3.4. Fluorescence microscopy

The tumors that were stained with Sirius red were then viewed on a Fluorescence Microscop (Leica, 20X, and LED-light) and then rendered on Progres C14\textsuperscript{plus}. The captured pictures were analyzed in Photoshop CS6\textsuperscript{®}.

4. Results

4.1. Choice of staining and slice

40 Slices were chosen stained with Sirius red. Hematoxylin &Eosin, as seen in figure 1, was disregarded because of the poor distinction between stroma and other tissue. Picro-mallory, as seen in figure 2, was disregarded because of the considerable background and difficult assessment in fluorescence microscope. Only the slices stained with Sirius red in saturated picric acid were further studied because of its clear fluorescence and distinguish of collagen compared to the other staining methods. Table 1 and 2 shows the different colors of tissue in tumor slices stained with H&E and Picro-mallory in optical microscope. Table 3 shows the different colors of tissue in tumor slices stained with Picro-mallory in fluorescence microscope and figure 3 shows the picture captured from the same microscope.

Table 1. Results of staining with H&E in optical microscope

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Blue</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Red</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>Red-pink</td>
</tr>
</tbody>
</table>
Figure 1. Tumor slice stained with H&E in optical microscope. Eosin is an outline stain which gives a clear reading between nuclei and cytoplasm. Hematoxylin colors the nuclei. Table 1 describes tissue coloring for this figure.

Table 2. Results of staining with Picro-mallory in optical microscope

<table>
<thead>
<tr>
<th>Tissue:</th>
<th>Color:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Collagen fibers</td>
<td>Dark blue</td>
</tr>
<tr>
<td>Fibroglia, neuroglia, axis cylinders and fibrin</td>
<td>Red</td>
</tr>
<tr>
<td>Myelin and erythrocytes</td>
<td>Yellow</td>
</tr>
<tr>
<td>Elastic fibers</td>
<td>Pale pink to yellow</td>
</tr>
</tbody>
</table>
Figure 2. Tumor slice stained with Picro-mallory in optical microscope. Components of connective tissue are selectively stained due to difference in affinity between dyes and tissue macromolecules. Table 2 describes tissue coloring for this figure.

Table 3. Results of staining with Picro-mallory in fluorescence microscope

<table>
<thead>
<tr>
<th>Tissue:</th>
<th>Color:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Red</td>
</tr>
<tr>
<td>Collagen fiber</td>
<td>Bright red</td>
</tr>
<tr>
<td>Other tissue</td>
<td>Red</td>
</tr>
</tbody>
</table>
Figure 3. Tumor slice stained with Picro-mallory in fluorescence microscope. Table 3 describes tissue coloring for this figure.

4.2. Sirius red in saturated picric acid slice pictures

In sirius-red in saturated picric acid the collagen is stained through sulfuric acid reacting with collagens basic groups. Table 4 shows the different colors of tissue in tumor slices stained with Sirius red.

Table 4. Results of staining with Sirius red in fluorescence microscope

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective Tissue, Collagen, basalmembran</td>
<td>Bright red</td>
</tr>
<tr>
<td>Every other tissue structure</td>
<td>Dark red</td>
</tr>
</tbody>
</table>

In the figures 4-7, the brighter strokes have been identified as stroma components possibly collagen and is the aim of this study to quantify.
Figure 4. Control tumor slice. Table 4 describes tissue coloring for this figure.
Figure 5. Tumor slice treated with SU11657 for 20 days. Table 4 describes tissue coloring for this figure.
Figure 6. Tumor slice treated with SU11657 for 40 days. Table 4 describes tissue coloring for this figure.
Figure 7. Tumor slice treated with Zoledronic Acid. Table 4 describes tissue coloring for this figure.

4.3. Change in stroma according to S/N values

Table 5. Changes in stroma according to S/N values

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>SU11657 (20 days)</th>
<th>SU11657 (40 days)</th>
<th>Zoledronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/N</td>
<td>2.85</td>
<td>3.11</td>
<td>4.06</td>
<td>3.02</td>
</tr>
<tr>
<td>% change</td>
<td>↑ 19 %</td>
<td>↑ 55 %</td>
<td>↑ 6 %</td>
<td></td>
</tr>
</tbody>
</table>

S/N values are the brightness difference between the stroma strokes S (Signal) divided with the brightness of the background tissue N (Noise). The S/N values for each treatment displayed in table 2 are a mean S/N value from ten slices each.
4.4. Change in tumor growth and angiogenesis

Table 6. Change in tumor growth and angiogenesis (Bäckman & Christofferson, 2005; Bäckman et al, 2008)

<table>
<thead>
<tr>
<th>Treatment:</th>
<th>Control</th>
<th>SU11657 (20 days)</th>
<th>SU11657 (40 days)</th>
<th>Zoledronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>% change in tumor growth</td>
<td>93 % ↓ in tumor growth</td>
<td>88.7 % ↓ in tumor growth</td>
<td>33 % ↓ in tumor growth</td>
<td></td>
</tr>
<tr>
<td>Qv (number of vessels/grid)</td>
<td>-89.5</td>
<td>-92.1</td>
<td>-39.7</td>
<td></td>
</tr>
</tbody>
</table>

5. Discussion

5.1. Angiogenesis

Folkman et al (1971) suggested that tumor growth and metastasis was angiogenesis dependent. With the absence of angiogenic factors tumors would be dependent on diffusion of nutrients from the extravascular space and be unable to grow more than a few millimeters (Folkman et al, 1971).

Tumor vessels are disorganized, heterogeneous and leaky. Endothelial cells in tumor vessels have also unusual features like expression of imbalanced surface molecules and being disorganized like the tumor vessels. These unique features could lead to selective inhibition. Several angiogenesis inhibitors have been found to inhibit tumor angiogenesis selectively without affecting the normal vasculature (Cao, 2004). Results have shown that inhibition of a single pro-angiogenic factor can delay tumor growth in experimental high risk NB. It’s suggested simultaneous inhibition of several angiogenic factors can impact angiogenesis on different levels with higher treatment efficacy as a result (Cao, 2004).
Neuroblastoma expresses angiogenesis stimulators and a correlation between increase in angiogenesis and poor outcome in neuroblastoma patients has been found. NB grows rapidly, is highly vascularized and often metastasizes. Anti-angiogenic might therefore improve long-term survival in patients with Neuroblastoma (Claffey & Robinson, 1996).

In the previous studies Bäckman & Christofferson, (2005); Bäckman et al, (2008) showed that angiogenic inhibitors, SU11657 and Zoledronic Acid could inhibit tumor growth in human NB tumor xenografted to mice as seen in table 6. SU11657 inhibits some of the most important receptors and mediators in angiogenesis, VEGFR, PDGFR, c-KIT, FLT3 (Bäckman & Christofferson, 2005). Zoledronic Acid is a member of the bisphosphonate class of compounds. The compounds have also shown to be inhibitors of angiogenesis and reduced the growth of cancer cell lines in vivo (Bäckman et al, 2008).

5.2. Stroma

Cancer cells are heterogeneous entities which interact with each other and the microenvironment in the vicinity. These interactions are dynamic and favor cell proliferation, movement and differentiations, while restricting cell death. Angiogenesis, cell migration and other remodeling properties are not unique to cancerous growth but are regulated programs normally operated under development or as a respond to acute tissue stress. A part of the microenvironment is the stromal compartment which provides contextual framework for organ or tissue but are activated in tumors and thus the response is different compared to normal stroma. The stromal cells often produce proteins as a response to wound healing or inflammation but during cancer development these proteins promote/favor cell proliferation, inflammation, angiogenesis and migration (Tlsty & Coussens, 2006).

The previous studies, Bäckman & Christofferson, (2005); Bäckman et al, (2008) studied only the angiogenesis aspect of SU11657 and Zoledronic acid. In this current study the impact on stroma was investigated. Since tumor stroma has shown that it plays
an important role in promoting tumor progression and metastasis, different stromal cells can be used as prognostic indicators (Conti & Thomas, 2011).

The parameter chosen to represent formation of new stroma in this study is collagen and the aim was to develop a reliable histological method to detect collagen or other stromal component. Collagen is a protein produced by activated stromal cells in tumors and was therefore a logical target for staining (Tlsty & Coussens, 2006).

But the irregularity of tumor vessels and its surrounding mural cells complicated the histological study of the slices (Cao, 2004). The staining with Sirius red in saturated picric acid and the microscopy in fluorescence simplified this problem. In the fluorescence microscope the stroma strokes are easily discerned against the background tissue. The other histological methods were disregarded either because of discernibility problems as in H&E where blood vessels and stroma were hard to tell apart. Picro-mallory was disregarded as mentioned earlier because of background issue in fluorescence microscope. Sirius red in saturated picric acid was chosen because of the simplicity in that connective tissue *i.e.* stroma/collagen was stained bright red and those stroma strokes had more fluorescence than the background which simplified quantification of collagen. The formation of collagen in the treated tumor slices were then compared to the untreated slices and quantified based on mean brightness (S/N) values. These values were taken manually in Photoshop CS6® from 10 slices for the control and each treatment. The Sirius red in saturated picric acid was also manually stained thus variability between each staining might occur. As such the reliability of the results may differ.

From prior studies, Bäckman & Christofferson, (2005); Bäckman *et al*, (2008), in table 6 indicates that tumor growth didn’t decline as much in the 40 day treatment with SU11657 compared to the 20 day treatment with SU11657 even though angiogenesis (Qv) decreased from SU11657 (20 days) to SU11657 (40 days). Coupled with the results from this study in changes of stroma, it could indicate that the expansion of stroma, according to S/N values, in SU11657 (40 days) could have led to tumor volume increase. Angiogenesis decreased for all the treatments regardless of length. These
results mirror quite well with the theory that tumor stroma is responsible for tumor proliferation and progression even in this case when angiogenesis is more or less absent. Therefore both stroma and angiogenesis should be inhibited to limit tumor growth.

Other methods for quantification of stroma could be the quantification of one of the stromal cells producing collagen such as Cancer associated fibroblast (CAF) which is the most prominent cell type within tumor stroma (Conti & Thomas, 2011).

CAFs stems from resident local fibroblasts, bone marrow-derived progenitor cells or transformed differentiating epithelial cells CAF can, through the provisions of various growth factors, hormones and cytokines, directly stimulate tumor cell proliferation. They produce insulin-like growth factor-1 and -2 which primarily helps tumors growth by imparting survival signals. CAF are also providers of extra-cellular matrix (ECM) components, such as various types of collagen, which are important for the malignant growth and invasion. CAF are documented to provide pro-angiogenic factors. They contribute to the tissue invasion and metastasis capabilities of cancers through inducing epithelial-to-mesenchymal transitions of tumor cells (Petrias & Östman, 2010). CAF are thus better markers for tumor stroma than collagen especially in regards to the highly metastatic NB if an appropriate histological method can be conducted.

5.3. Conclusion

The increase in tumor volume, decreased number of vessels and expansion of tumor stroma in the tumors in the long-term treatment with SU11657 indicates that the tumors survive the angiogenic-treatment through an expansion/activation of its stroma. This needs to be further evaluated through a quantification of the activity of the organelles and/or cells in the stroma compartment such as cancer-associated fibroblast and other inflammatory cells. The histological staining with Sirius red in saturated picric acid marked collagen better than previous tried histological methods and enabled quantification of stroma.
6. Reference


