Wound Repair and Angiogenesis in Skin


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In skin there is avascular tissue (epidermis) overlying richly vascularized tissue (dermis) that also contains many lymphatics. Two major topics that will be addressed are (1) the role blood vessels as important targets for anti-inflammatory therapy and (2) recent aspects of tumor lymphangiogenesis and in particular a new concept of lymph node lymphangiogenesis and its contribution to tumor spread.

Psoriasis is an inflammatory skin disease with unknown etiology characterized by epidermal hyperplasia consisting of finger-like projections, chronic inflammation, and pronounced angiogenesis. VEGF is highly upregulated in the epidermis and VEGF receptors are upregulated in the dermis. There are two independent mechanisms of angiogenesis in psoriasis. (1) Epidermal keratinocytes hyper-proliferate in psoriasis (but also in wound healing and squamous cell carcinoma) and they release growth factors of the EGF family such as TGFα or amphiregulin, which in an autocrine way stimulate the cells to release VEGF and PlGF. The VEGF and PlGF then stimulate cutaneous vessels. This mechanism makes sense, because whenever you have proliferation of an avascular tissue, you need a way to ensure enhanced vascular support. (2) Direct hypoxia-induced release of VEGF and PlGF.

So VEGF and PlGF are upregulated in psoriasis, but can the phenotypic characteristics of psoriasis be induced by persistent activation of the dermal vessels? Transgenic mice that over-express PlGF or VEGF under control of the K14 promoter were generated. These mice did not develop the chronic inflammation of psoriasis. The major phenotype was enhanced acute inflammation. In a delayed type hypersensitivity model, mice are made allergic and then challenged leading to inflammation and edema of the ear. The swelling of the ear is measured as a parameter of the amount of inflammation. In a normal mouse, there is initial inflammation that peaks at 48 hours and within a week it resolves. In PlGF overexpressors, there was increased inflammation and edema, but just as in normal mice, it did not persist. In contrast, in VEGF overexpressors, there is persistent inflammation; they are completely unable to downregulate inflammation once it has been induced. The epidermis becomes thicker and has fingerlike projections something like that seen in psoriasis. Homozygous VEGF transgenic mice develop spontaneous inflammatory lesions at 6 months of age that look very much like psoriasis with inflammation, induration, scaly skin. Histologically they are indistinguishable from psoriasis with hyperplastic epidermal keratinocytes with fingerlike projections, inflammation with CD4 cells in the dermis and CD8 cells in the epidermis, neutrophils, and prominent angiogenesis. This suggests that chronic overexpression of VEGF in the skin is sufficient to generate a psoriasis phenotype. It is also necessary to maintain the disease, because treatment with VEGF-trap results in normality within 2 weeks.
There is also genetic data implicating VEGF in psoriasis. The *Vegf* gene is located near the major psoriasis susceptibility locus. There have been several SNPs described in the VEGF gene including a G to C polymorphism at 634 in the 5’ UTR, which has been reported to be associated with enhanced VEGF levels in normal individuals and the frequency of this genotype is significantly increased in psoriasis patients that have severe disease. There are certain SNPs or haplotypes that can predict whether a patient will respond to retinoid therapy. The hypothesis is that there is an angiogenic constitution that might determine susceptibility to chronic inflammation and also response to retinoids. Does that apply just to the skin disease or to other chronic inflammatory diseases as well? VEGF is not the only agent that contributes. The VEGF transgenics were crossed with PlGF KO mice. Mice that are VEGF transgenic and wild type for PlGF have persistent inflammation in the skin, while VEGF transgenics that lack PlGF have inflammation that is a bit prolonged compared to wild type mice, but it goes down to background levels within 2-3 weeks. There are 2 SNPs in the PlGF gene that are associated with severe psoriasis and there is a possibility of genetic interaction between the VEGF and PlGF genes in patients with psoriasis. There are certain combinations of SNPs that are significantly increased in early onset or more severe psoriasis.

So both VEGF and PlGF may be good targets for treatment in psoriasis and other chronic inflammatory diseases. Inflammation was induced in the skin of wild type mice and then they were treated systemically with an antibody directed against VEGF receptor 1 or 2 (Imclone), or both together. Either antibody alone did not have a significant effect, but both together significantly inhibited the inflammatory response. Blocking lymphangiogenesis in this model is also beneficial.

**How tumors metastasize to lymph nodes**

The traditional model of tumor progression is that tumors induce angiogenesis to promote their growth and this increases metastasis, but how do tumor cells get into lymph nodes? The dogma has been that lymphatic vessels play a passive role and tumor cells happen upon these pre-existent lymphatics and then are washed to the lymph nodes. It was almost impossible to image the lymphatic vessels because markers weren’t available and lymphangiogenic factors were unknown. Now it is known that at a certain stage lymphatics develop from pre-existent embryonic veins. The transcription factor PROX1 is switched on by unknown stimuli, and this results in cells budding off from the veins and migrating and during this process the cells lose expression of blood vessel-specific genes and begin expressing lymphatic-specific markers: live1, VEGF receptor 3, and PROX1.

Lymphatic endothelial cells can be cultured and they maintain their lineage-specific differentiation over many passages. Transcriptional profiling of these cells compared to vascular endothelial cells can help to reveal other lymphatic-specific genes. Both the Affimetrix platform and Applied Biosystems Gene Array platform gave comparable results. Some previously known genes such as VEGF receptor 3 and PROX1 were upregulated, but many novel genes were also upregulated.

Vascular endothelial cells can be reprogrammed by infection with Kaposi sarcoma virus carrying lymphatic-specific genes. VEGF-C and -D are relatively specific lymphangiogenic growth factors that work through VEGF receptor 3 on lymphatic endothelial cells.

In an orthotopic breast cancer model, lymphatic vessels can be seen to proliferate and spread into tumors. The degree of lymphangiogenesis correlates with the amount of metastasis, suggesting it might be a prognostic feature and a therapeutic target. It has been suggested that
this could be an artifact of xenografts and that human tumors do not have lymphangiogenesis. Malignant melanomas metastasize very early to lymph nodes. Blood vessels occur homogenously throughout tumors, but lymphangiogenesis occurs in hot spots. Lymphangiogenesis seems to be a sensitive prognostic feature for future metastasis of melanomas. Primary tumors were graded for amount of lymphangiogenesis and three groups were identified: (1) no lymphangiogenesis, (2) abundant lymphangiogenesis, and (3) moderate lymphangiogenesis. The results were striking. None of the group 1 patients developed lymph node metastasis over the next 10 years and were still alive, compared to metastasis to lymph nodes in all of the patients in group 2 and only 10-15% survived at 10 years. So lymphangiogenesis does occur in humans and has prognostic significance.

VEGF-C and -D can induce lymphangiogenesis mostly surrounding tumors but sometimes inside of tumors. The big question mark is the role of VEGF-A in this process; it has been considered just an angiogenic factor, but lymphatic vessels express VEGF receptor 2.

Transgenic mice that express GFP under control of the K14 promoter have green skin. When skin cells become carcinogenic in a skin carcinogenesis model, they remain fluorescent, and they are still fluorescent after metastasis. It is very easy to detect metastasis and can also homogenize lymph nodes and quantify metastasis. The GFP mice were crossed with VEGF transgenics. In wild type mice, angiogenesis occurs very early in benign tumors and it was enhanced when VEGF was overexpressed, but what was surprising was that there were many more lymphatics in the tumors and they were proliferating as shown by BRDU labeling. So VEGF-A is a lymphangiogenic agent. All of the VEGF overexpressors developed metastasis to regional lymph nodes. These VEGF-producing tumor cells continue to promote lymphangiogenesis in the lymph nodes, which results in more metastasis to distant sites. The tumors induce lymphangiogenesis in the draining lymph nodes even before they metastasize, which is a new twist to the seed and soil hypothesis. The seed can actively modify the soil to enhance the chances of metastasis. So there is lymphangiogenesis in the primary tumors promoting metastasis to the sentinel lymph nodes where the tumor cells can cause more lymphangiogenesis promoting metastasis to distant lymph nodes and organs.

Questions

1. Can you exclude two confounding variables in the VEGF-A skin carcinogenesis model? You showed previously that VEGF-A leads to chronic inflammation and in the carcinogenesis model you paint with TPA, which is a very strong irritant and causes the release of many inflammatory agents. It would be expected that the VEGF-A mice would have much more inflammation and inflammatory cells are sources of lymphangiogenic stimulators (VEGF C and other agents). Secondly, in the metastasis when you have VEGF-A producing tumor cells, which grow faster, how can you separate the role of lymphangiogenesis from the advantage that these cells have in metastasis.

For the first question, TPA does induce some inflammation. We looked at inflammatory cells in tumors from VEGF-A transgenics and wild type mice and we didn’t see major differences in CD11b-positive macrophages, CD4-positive lymphocytes, and others. We also performed in situ hybridization for VEGF-C and –D and the major source of VEGF-C was tumor cells or epidermal keratinocytes, but do not see any difference in stromal cells or inflammatory cells VEGF-C in VEGF-A overexpressing mice compared to wild type. So with the tools that
we have, we cannot see that there is enhanced inflammation in the VEGF-A overexpressors in this model.

For the second question, when the cells are cultured, the VEGF-A expressing tumor cells do not proliferate faster than the wild type tumor cells. When you look at these lymph nodes containing metastases and you see these induced lymphatic channels inside of lymph nodes, it is suggestive that it may facilitate distant spread.

2. You showed that lymphatic density predicts melanoma progression. Is that independent of vessel density?
   Yes. We did multivariant analysis.

3. If you treat melanomas with VEGF inhibitors, you would predict that lymphatics would also respond- what have you seen in that setting?
   In fact, melanoma may not be very VEGF-dependent. It may be more driven by FGF2 or other factors. We don’t think the lymphangiogenesis in melanoma is VEGF-A driven. We think there is another lymphangiogenic factor overexpressed in melanoma.