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Requirement of lipocalin 2 for hematopoietic and solid tumor malignancies

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Introduction

The lipocalin 2 gene encodes a secreted glycoprotein involved in iron binding, stabilizing metalloproteinase MMP-9, and induction of apoptosis. Our previous studies first showed that soft agar clones of a CML cell line (K562 cells) caused atrophy of spleen and marrow cells and a wasting syndrome in some of the leukemic mice, leading to early death (Lin *et al.*, 2001). In later studies, we found that Bcr-Abl⁺ mouse hematopoietic cells and agar clones of CML cell line K562 secrete lipocalin 2, and that conditioned medium from these cells induces apoptosis in target bone marrow cells (Lin *et al.*, 2005 and Leng *et al.*, 2008). We also found that reducing lipocalin 2 expression in mouse Bcr-Abl⁺ cells by either anti-sense or siRNA knockdown strongly reduces over-growth of BCR-ABL⁺ mouse myeloid 32D cells in marrow and spleen of NOD/scid mice (Lin *et al.*, 2005).

To explore further the role of lipocalin 2 in BCR-ABL induced leukemia, we used the mouse transplant model, which causes a lethal myeloid leukemia involving enlarged spleens, liver and marrow and blood invasion in recipient mice receiving retroviral infected BCR-ABL⁺ bone marrow cells. Consistent with our previous findings (Lin *et al.*, 2005), we found that donor marrow cells expressing BCR-ABL but lacking lipocalin 2 (24p3) (obtained from 24p3 null mice) did not cause leukemia or any disease in non-irradiated wild type recipient mice.

Cultured bone marrow cells expressing BCR-ABL from wild-type mice as expected caused solid tumors in nude mice following s.c. injection of marrow cells whereas BCR-ABL⁺ bone marrow cells from lipocalin 2 null mice did not cause tumors, indicating that lipocalin 2 expression is also required for tissue invasion and solid tumor formation. These findings demonstrate that lipocalin 2 has two critical functions in tumor formation: one involving over-growth in marrow and spleen tissues in leukemia and the other involving invasion of tissues needed for solid tumor formation and metastasis.

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Materials and Methods

The methods, laboratory procedures, animal experiments were described in papers by Lin *et al.* (2001), Lin *et al.* (2005) and Leng *et al.* (2008).

Results and Discussions

Mouse transplant leukemia studies: Lipocalin 2 is required for leukemia

We examined the role of lipocalin 2 in the mouse transplant model. This model is considered to be the best model for mimicking chronic myeloid leukemia. Our findings show that Bcr-Abl+ marrow cells from lipocalin 2 knock-out mice were completely ineffective for causing leukemia under conditions of transplant in which the recipient marrow and spleen tissue were intact (no preconditioning with irradiation) (Fig. 1 a–b). However, if mice were irradiated before transplant, marrow cells from 24p3 knock-out mice regained their ability to cause leukemia when inoculated at high dose (Fig. c–d). Liver, spleen and marrow tissues from these respective mice completely supported these findings (Leng *et al.*, 2008). Additional studies and controls are published in our recent Oncogene paper (Leng *et al.*, 2008).

Studies with CML cell line K562 with lipocalin 2 over-expression

Over-expression of lipocalin 2 in the CML cell line K562 increases the level of apoptosis in the spleen of nude mice injected with K562 cells over-expressing lipocalin 2, as measured by cleaved Caspase 3 expression (Fig. 2). These findings are consistent with our earlier studies showing that conditioned media from Bcr-Abl+ cells induces apoptosis in mouse hematopoietic cells lacking Bcr-Abl (Lin *et al.*, 2005). They are also consistent with our findings in NOD/scid mice injected with agar clones of K562 cells, which express higher levels of lipocalin 2 than uncloned K562 cells, showing that these mice develop a wasting syndrome and atrophy of marrow and spleen tissues (Fig. 3) (Lin *et al.*, 2001, Leng *et al.*, 2008).

Lipocalin 2 required for solid tumor formation caused by Bcr-Abl+ marrow cells

Our studies in breast cancer cell lines indicated that lipocalin 2 is involved in tissue invasion (see below). Therefore, since marrow cells expressing Bcr-Abl cause solid tumors by s.c. injection of nude mice, we performed experiments to determine whether marrow cells expressing Bcr-Abl from lipocalin 2 knock-out mice retain these effects. The results show that Bcr-Abl+ marrow cells form solid tumors and importantly Bcr-Abl+ marrow cells lacking lipocalin 2 expression do not (Fig. 4). Intravenous injection of wild type Bcr-Abl positive marrow cells caused solid tumors in nude mice but lipocalin 2 knock-out Bcr-Abl cells did not have this activity (Leng *et al.*, 2008).

Lipocalin 2 is a critical player in breast cancer formation in mice

Our preliminary studies indicate that lipocalin 2 expression in breast cancer cell lines and breast cancer patients correlates with HER2/neu over-expression (T. Ding, X. Leng, Y. Wu and Arlinghaus in preparation). Also, studies with lipocalin 2 knock-out mice indicate that breast tumor formation is significantly delayed in these mice in the presence of Her2/neu (V664E) transgene. Thus, lipocalin 2 is not just a marker for breast cancer, rather it is a critical player for breast tumor formation. Human breast cancer cell lines require lipocalin 2 expression for increased invasion and increased growth in soft agar. Knockdown of lipocalin 2 expression reduced all of these properties and reduced breast tumor formation in xenograph model (T. Ding, X. Leng, Y. Wu and Arlinghaus in preparation).

Our findings indicate that lipocalin 2 plays an important role in leukemia and breast cancer. What are the mechanisms involved? Lipocalin 2 is known to bind and carry small molecules like siderophores (Goetz *et al.*, 2002), and to stabilize metalloproteinase MMP-9 (Yan *et al.*

2001). We believe that both mechanisms play a role in the oncogenic effects of lipocalin 2. Studies by the Michael Green lab have proposed that lipocalin 2 causes cell death in normal hematopoietic cells by depletion of still unknown siderophore, leading to induction of apoptosis (Devireddy *et al.* 2001, 2005). Our findings with lipocalin 2 in CML indicate that secretion of lipocalin 2 by CML cells is absolutely required to over-grow leukemia cells in marrow and spleen tissues of mice, since leukemia cells lacking lipocalin 2 secretion cannot out compete for tissue space in marrow and spleen in a robust normal tissue environment (Leng *et al.*, 2008). Lacking lipocalin 2 function in the surrounding medium of tissues invaded by leukemia cells should allow killer T cells to destroy the leukemia cells. Thus, strategies that neutralize lipocalin 2 effects should be useful in therapy of CML. We have proposed a model that illustrates how a few leukemia cells utilize lipocalin 2 to invade the marrow and spleen and to over-grow in these tissues at the expense of dead or dying normal hematopoietic cells (Fig. 5). As leukemia cells leave the marrow environment, we propose that lipocalin 2 secretion will assist and facilitate the invasion of other tissues like liver, kidney and spinal cord that is seen in terminal stages of CML patients.

These invasive effects of persistent and continuous secretion lipocalin 2 by breast cancer cells and for that matter any metastatic tumor cell will facilitate expansion of the tumor mass and metastatic spread of the tumors. Interesting and novel mechanism(s) concerning the action of lipocalin 2 in metastatic breast cancer will likely be discovered in the next few years, which will aid in developing new and more effective strategies to treat cancer.

Summary

Our published studies indicate that lipocalin 2 is a critical factor for invasion of hematopoietic tissues by chronic myelogenous leukemia cells. Lipocalin 2 is secreted by Bcr-Abl+ chronic myeloid leukemia cells causing apoptosis in normal hematopoietic cells but not in Bcr-Abl+ cells. Secreted lipocalin 2 forms a complex with metalloproteinase MMP-9, and is reported to stabilize its activity, which implies that lipocalin 2 may have a role in solid tumor formation. Our studies indicate that Bcr-Abl+ mouse marrow cells lacking the lipocalin 2 gene are not only defective for leukemia induction but also are defective in forming solid tumors compared to Bcr-Abl marrow cells that express lipocalin 2. Thus, our findings indicate that lipocalin 2 has two functions in tumor malignancies: one involving induction of apoptosis in normal hematopoietic cells and the other to facilitate solid tumor formation.

Acknowledgments

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Fig.1.

24p3 is required for BCR-ABL-mediated leukemia induction in the mouse BMT model. BCR-ABL+ marrow cells from 24p3KO mice are unable to induce disease in non-irradiated recipient mice when receiving either high dose (~1million/recipient) (**a**) or low dose (~0.25million/recipient) (**b**) donor cells. (**c**) BCR-ABL+ marrow cells from 24p3KO mice cause minor disease in irradiated recipient mice (600 cGy) injected with a low dose of donor cells. Transplantation of a high dose of marrow cells induces leukemia in irradiated recipient mice (600 cGy) regardless of whether the marrow cells are derived from 24p3 KO mice or WT mice (**d**).

Fig.2. Overexpression of NGAL in K562 causes suppression of hematopoiesis (“at” marked as atrophy) and increased rate of apoptosis in the recipient nude mice (250 cGy) as measured by cleaved Caspase 3 expression.

Fig.3. Survival curves of irradiated (250 cGy) NOD/scid mice injected with K562, C5 (high NGAL expression) and C6 cells (low NGAL expression) (1×10^6).

Fig.4.

24p3 expression is required for solid tumor formation. BCR-ABL+ marrow cells from 24p3 knock-out mice do not cause solid tumors when s.c. inoculated in nude mice. Mice were injected with either 0.5×10^6 or 2×10^6 of Bcr-Abl positive marrow cell lines, wild type on the left and 24p3 knock-out on the right, as indicated.

Fig.5.

A model for the role of NGAL in the expansion of BCR-ABL+ cells within the marrow of early stage CML patients. NGAL is illustrated by the brown round circles; The Y like structure on the surface of normal hematopoietic cells represents the receptor for NGAL. The skull and cross bone indicates dying normal hematopoietic cells; Ph+ marks Philadelphia chromosome positive cells.