Original Article

Hepcidin Expression in the Liver of Mice with Implanted Tumour Reacts to Iron Deficiency, Inflammation and Erythropoietin Administration

(hepcidin / anaemia / tumour / inflammation / erythropoietin)

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Abstract. Cancer is known to be an important cause of anaemia due to several factors including iron deficiency and inflammation. Hepcidin, a key regulator of iron metabolism, is up-regulated by iron and inflammatory stimuli such as interleukin 6, and decreased by iron deficiency, enhanced erythropoiesis and hypoxia. It is supposed to play a crucial role in changes of iron metabolism in anaemia of chronic disease, which is characterized by sequestering iron in macrophages and decreasing its availability for red blood cell production. To study the effect of tumour growth on hepcidin expression, we implanted human melanoma cells into mice and studied the changes of the amount of liver hepcidin mRNA by real-time PCR. We observed development of anaemia, which correlated with the size of the tumour. Hepcidin expression significantly decreased with the anaemia development, but in late stages we observed an increase of its expression together with an increase of mRNA for interleukin 6. However, the increase of hepcidin expression could be inhibited by exogenous erythropoietin administration. In our model of tumour growth, hepcidin expression reflected anaemia development and iron deficiency, erythropoietin administration and inflammation, and we suppose that it could therefore serve as a useful marker of these clinical situations common in cancer patients and play a role in the pathogenesis of cancer-associated anaemia.

Introduction

Anaemia is a common symptom in patients with tumours and has a complex pathogenesis (Grotto, 2008). Apart from treatment-related factors there are several important disease-related factors such as blood loss, iron, vitamin and nutrient deficiency, haemolysis, infiltration of bone marrow, and inflammation. Iron deficiency is mainly caused by iron consumption by tumour cells, bleeding, and insufficient nutrition or impaired gastrointestinal absorption.

Inflammation is an important factor of anaemia of chronic disease (ACD), also called anaemia of inflammation (Weiss and Goodnough, 2005). Its pathogenesis results from activation of the immune system and activation of inflammatory mediators and cytokines, mainly interleukin (IL) 6, 1, tumour necrosis factor α (TNF-α), interferon γ (IFN-γ). It is characterized by decreased red blood cell survival, decreased erythropoietin (EPO) production and EPO resistance. Erythropoietin is frequently administered to treat anaemia in cancer; however, many questions about its use remain to be answered (Adamson, 2008). Moreover, important dysregulation of iron metabolism is present in ACD (Theurl et al., 2009). Iron is sequestrated in macrophages and its availability for erythropoiesis is decreased (Ganz and Nemeth, 2009). This dysregulation of iron metabolism is supposed to be mediated by increased expression of hepcidin (Weinstein et al., 2002).

Hepcidin is a key regulator of iron metabolism (Nemeth and Ganz, 2009). It is a 25-amino acid antimicrobial peptide encoded by the *Hamp* gene and produced mainly by hepatocytes. Hepcidin decreases iron resorption in the duodenum and inhibits its release from mac-

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Abbreviations: ACD – anaemia of chronic disease, BMP – bone morphogenetic protein, BMPR – bone morphogenetic protein receptor, DMT-1 – divalent metal transporter 1, EPO – erythropoietin, FPN – ferroportin, HJV – hemojuvelin, IFN – interferon, IL – interleukin, PCR – polymerase chain reaction, STAT – signal transducer and activator of transcription, TfR – transferrin receptor, TNF – tumour necrosis factor.

rophages (Nicolas et al., 2001). On these cell types it interacts with ferroportin, the only known cellular iron exporter, and causes its internalization and degradation (Nemeth et al., 2004b).

Hepcidin expression is regulated by the iron status, erythropoiesis, hypoxia and inflammation (Nicolas et al., 2002a). The molecular mechanisms of this control have been at least partially elucidated (Zhang and Enns, 2009). While iron deficiency, enhanced red blood cell production and hypoxia decrease its expression to accelerate iron absorption, iron overload and inflammatory stimuli cause increased hepcidin expression. It is mainly IL-6, an acute-phase cytokine, which causes a significant increase of hepcidin and hypoferraemia (Nemeth et al., 2004a; Kemna et al., 2005). Hypoferraemia that is supposed to be beneficial in infection can lead to iron-restricted erythropoiesis and is considered as one of the pathogenetic mechanisms of ACD (Weiss, 2009). IL-6 induces hepcidin expression through STAT3-dependent mechanisms (Wrighting and Andrews, 2006). The erythropoietic regulatory pathway includes growth differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1) (Tanno et al., 2007; 2009). Regulation by iron is complex and includes several iron-sensing proteins expressed in hepatocytes (Nemeth and Ganz, 2009). A critical role is played by bone morphogenetic protein (BMP) signalling, which results in phosphorylation of cytoplasmic Smads and activation of Smad4, translocating to the nucleus as a transcription factor. This signalling is modulated by BMPR co-receptor called haemojuvelin (HJV), which in turn is further regulated by matriptase 2. In addition to this pathway, transferrin saturation controls hepcidin expression through the interaction with transferrin receptor 1 (TfR1) and subsequent association of TfR2 with HFE, which leads by a so far unknown mechanism to *Hamp* activation. Combination of different stimuli and their subcellular signal pathways results in hepcidin transcription and expression in various clinical situations (Kemna et al., 2008).

The role of hepcidin in cancer is of interest because of its presumed pathogenetic importance in the development of anaemia in this disease and because of the supposed importance of its measurement in the diagnostic process. Several clinical studies have been published in recent years about hepcidin in cancer patients. It has been studied in renal cell carcinoma (Kamai et al., 2009), colorectal carcinoma (Ward et al., 2008), multiple myeloma (Sharma et al., 2008) or lymphoma (Hohaus et al., 2010). So far, there is no clear answer about the role of hepcidin in anaemia in patients with cancer. This may be due to the large variability of this disease and partially due to different methods used for hepcidin determination. To date little experimental data have been published.

To study the possible role of hepcidin in tumour-related anaemia we implanted human melanoma cells into mice (Pouckova et al., 1998) and measured the changes in the expression of hepcidin and other iron-related genes in the liver.

Material and Methods

Animals

All studies were conducted using male mice C57BL/6N (Charles River, Wilmington, MA), age ranging from 2 to 3 months. The animals were maintained in a temperature- and light-controlled environment. They had free access to tap water and standard laboratory food. Control animals were subjected to experimental manipulations similar to those of treated mice. The animals were sacrificed at different intervals after the tumour administration: day 2, day 7, day 15, day 18 and days 20–23 (pooled as days 20+) and a part of the liver tissue was removed and placed in RNAlater solution (Sigma-Aldrich, St. Louis, MO).

Human melanoma tissue (gift from Dr. Poučková from the Institute of Biophysics and Informatics of the First Faculty of Medicine, Charles University, Prague) was suspended in saline and administered subcutaneously in the interscapular region (Pouckova et al., 1998). The control group was injected with saline only. Four experiments were performed and the final number of animals in the groups varied from 5 to 11.

The Animal Care Committee of the First Faculty of Medicine approved the experiments.

Erythropoietin (EPO) administration

Human recombinant erythropoietin (Eprex®, Cilag AG, Schaffhausen, Switzerland) diluted in saline (50 U/mouse) was administered subcutaneously on four consecutive days prior to the liver removal.

RNA isolation and reverse transcription

Total RNA was extracted from the tissues using RNABlue (Top-Bio, Brno, Czech Republic). The tissue was homogenized in RNABlue and RNA was extracted using chloroform/isopropanol/ethanol. Its concentration and purity was measured spectrophotometrically at wavelength 260 and 280 nm. First-strand cDNA synthesis was performed in a total volume of 20 µl containing 200 U of M-MuLV reverse transcriptase, 4 µl of 5× Reaction Buffer, 20 U of RNase inhibitor, 2 µl of Deoxynucleotide Mix (final 1 mM), 1 µl of oligo(dT)$_{18}$ primer (0.5 µg), and 1 µg of total RNA, as recommended by the manufacturer (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas, Thermo Fischer Scientific, Waltham, MA).

Real-time PCR

Gene expression studies were performed in a Roche LightCycler™ real-time PCR instrument, using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (Vokurka et al., 2006). Values are represented as the amount of target mRNA relative to β-actin. Primer sequences were: **β-actin** forward 5'-GAC ATG GAG AAG ATC TGG CA-3', reverse 5'-GGT CTT TAC GGA TGT CAA CG-3'; **hepcidin** for-

ward (common hepcidin primer) 5'-CTG AGC AGC ACC ACC TAT CTC -3', reverse 5'-TGG CTC TAG GCT ATG TTT TGC -3'; **hepcidin 2** reverse 5'-GGC TCT AAG CTC TCT ATT CTT CA -3'; **hemojuvelin** forward 5'- CCC AGA TCC CTG TGA CTA TGA -3', reverse 5'- CAG GAA GAT TGT CCA CCT CAG -3'; **DMT1-IRE** forward 5'- CAA TGG AAT AGG CTG GAG GAT -3', reverse 5'- ACA GAC CCA ATG CAA TCA AAC -3'; **ferroportin** forward 5'- TCG GTT CCT CTC ACT CCT GT -3', reverse 5'- GTG GAG AGA GAG TGG CCA AG -3'; **TfR1** forward 5'- TGG GTC TAA GTC TAC AGT GGC -3', reverse 5'- AGA TAC ATA GGG CGA CAG GAA -3' ; **TfR2** forward 5'- ATT CTC CTT TCT CCC TCT TT -3', reverse 5'- GCT GTC CAT CTC ACT CTC TA -3'; **BMP-6** forward 5'- GAA CCT GGT GGA GTA CGA CAA 3', reverse 5'- ATG CTC CTG CAA GAC TTG GTA -3'; **IL-6** forward 5'- AGT TGC CTT CTT GGG ACT GA- 3', reverse 5'- TCC ACG ATT TCC CAG AGA AC -3'.

Haematological analysis

Mice were exsanguinated under halothane anaesthesia from axillary blood vessels. Haematological parameters were measured using Advia™ 60, Hematology Systems (Bayer, Tarrytown, NY).

Iron determination

Plasmatic iron concentration was measured in a routine biochemical laboratory.

Liver non-haem iron was determined according to the method of Torrance and Bothwell (Torrance and Bothwell, 1980) and expressed per wet weight of tissue.

Statistical analysis

Multiple comparisons were evaluated by the one-way analysis of variance (ANOVA) followed by the Tukey test. Student's upaired test was used for comparison between two groups. Pearson test was used for correlation calculations. Values in the figures are expressed as means ± standard error of the mean (S.E.M.). Significance was accepted at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 4.

Results

Anaemia developed with the tumour growth

Injection of melanoma cells into mice resulted in tumour growth and anaemia development. Haematocrit decreased significantly from the 15th day after the tumour administration (Fig. 1A). Its value correlated negatively with the tumour growth expressed as percentage of the tumour size to the total body size (Fig. 1B). Sizes of the spleen gradually increased from 116.9 ± 14.9 mg in control mice and reached statistical significance on day 15 (185.3 ± 24.1 mg) and day 18 (248.5 ± 46.9 mg). The size of the spleen negatively correlated with haematocrit ($r = -0.4034$, $P = 0.0054$). The liver iron content did not change between the control group and the group of mice with tumours on days 20+ (45.3 vs. 47.9 μg/g wet tissue). Serum iron concentration differed significantly between controls and animals on day 15 (25.9 ± 1.1 vs. 12.9 ± 1.4 μmol/l, $P < 0.05$); however, on days 20+ the iron concentration increased to 18.6 ± 2.2 μmol/l.

Expression of hepcidin, IL-6 and other iron-related genes in the liver

Hepcidin mRNA expression in the liver insignificantly increased on the second day after tumour administration and then was gradually decreasing. The decrease reached statistical significance on day 18 (Fig. 2A) and in this interval hepcidin expression was negatively correlated with haematocrit (Fig. 2B). However, in later stages (days 20+) hepcidin mRNA insignificantly increased to initial levels despite persisting and even worsening anaemia. Hepcidin 2 mRNA decreased in a similar mode on day 18; however, it remained decreased on days 20+ (Fig. 2C).

IL-6 mRNA expression in the liver was significantly increased on days 20+ compared to all other intervals (Fig. 3A). IL-6 expression correlated with hepcidin expression (Fig. 3B); however, when these correlations were calculated at different intervals, the correlation was significant only on day 2 ($r = 0.9354$, $P = 0.0061$) and days 20+ ($r = 0.8301$, $P = 0.0056$).

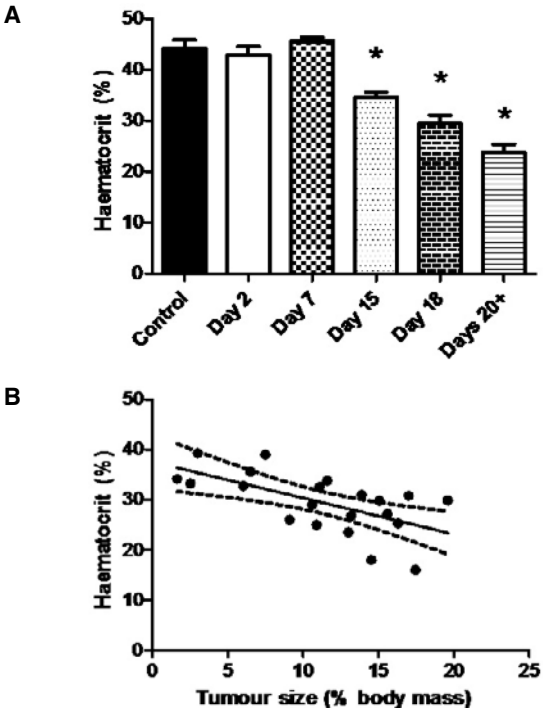

Fig. 1. Development of anaemia in mice with implanted tumours. (A) Changes in the haematocrit at different intervals after tumour implantation – *) statistically significant in comparison with the control group ($P < 0.05$). (B) Correlation between tumour size (expressed as percentage of the tumour size to the total body mass) and haematocrit ($r = -0.6213$, $P = 0.0026$).

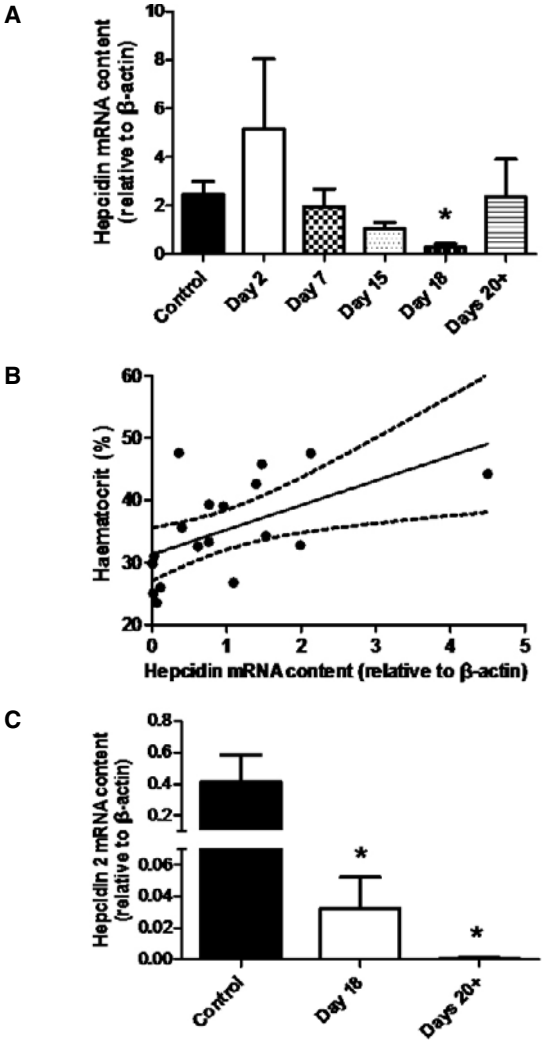

Fig. 2. Changes in hepcidin expression in the liver of mice with implanted tumours at different intervals after tumour implantation. (A) The hepcidin mRNA amount significantly decreased on day 18 in comparison with the control group ($P < 0.05$). (B) Correlation between hepcidin expression (expressed as a ratio to β-actin) and haematocrit in mice on days 7–18 ($r = 0.5609$, $P = 0.0125$). (C) Hepcidin 2 measured on days 18 and 20+ decreased significantly on both days compared to the control group ($P < 0.05$).

From the other studied genes, *BMP6* (Fig. 4A) and *HJV* (Fig. 4B) were decreased on day 18; the expression of other studied genes was not statistically changed (Fig. 4C).

Administration of erythropoeitin decreased hepcidin expression in anaemic mice with tumours

Four-day administration of erythropoietin from the day 18 caused a decrease of hepcidin expression (Fig. 5A) on day 22. This decrease was also observed for hepcidin 2 (data not presented) and was accompanied by a distinct but insignificant decrease of IL-6 expression in the liver (Fig. 5B). The mean size of the spleen increased after the EPO administration from 179.2 ± 13.8 mg to 349.4 ± 8.6 mg ($P < 0.0001$); however, the haematocrit did not change significantly within this period.

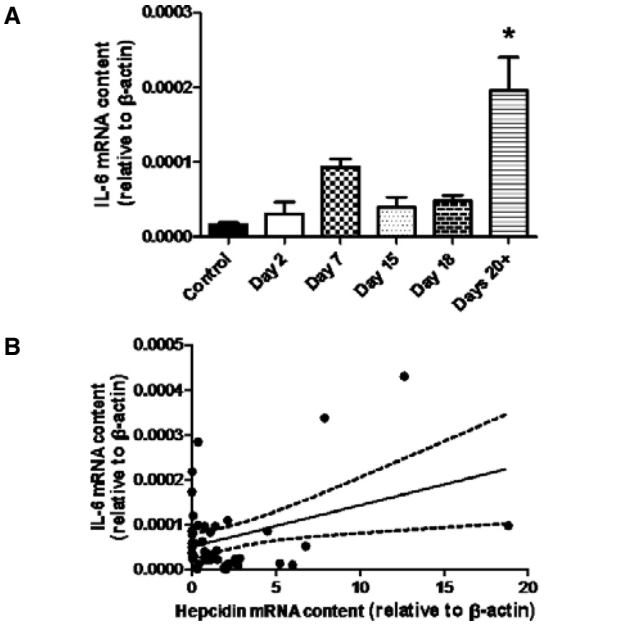

Fig. 3. Measurement of interleukin 6 mRNA in the liver of mice with implanted tumours. (A) Changes in interleukin 6 expression at different intervals after tumour implantation. *) Significantly different from all other groups ($P < 0.05$). (B) Correlation between hepcidin expression (expressed as a ratio to β-actin) and interleukin 6 mRNA ($r = 0.3677$, $P = 0.0130$).

Discussion

In this paper we describe the administration of human melanoma cells to mice in order to study hepcidin mRNA expression in the liver. The mice showed distinct anaemia development correlated to tumour growth and a significant decrease of hepcidin expression. Only at late stages with significant tumour size and necrosis, hepcidin expression tended to increase to initial levels. This increase correlated with the increase of IL-6 mRNA in the liver and could be prevented by EPO administration.

The regulation of hepcidin expression has been partially elucidated in the last years, and the impact of different clinical stimuli such as iron, erythropoiesis and inflammation on hepcidin expression and concentration are of major interest in both experimental and clinical studies (Kemna et al., 2008). Apart from its pathogenetic role, hepcidin can serve as a clinical marker of these conditions. It can reflect changes of iron metabolism and inflammation that may develop at different stages of tumour growth.

In our model we propose that the decrease of hepcidin expression was caused by the fast growth of the tumour and intratumoral bleeding which led to anaemia, stimulation of erythropoiesis and increased requirements for iron. The size of the spleen, an important site of haemopoesis in mice, was increased in anaemic mice. We did not measure hepcidin and iron content in the tumour as

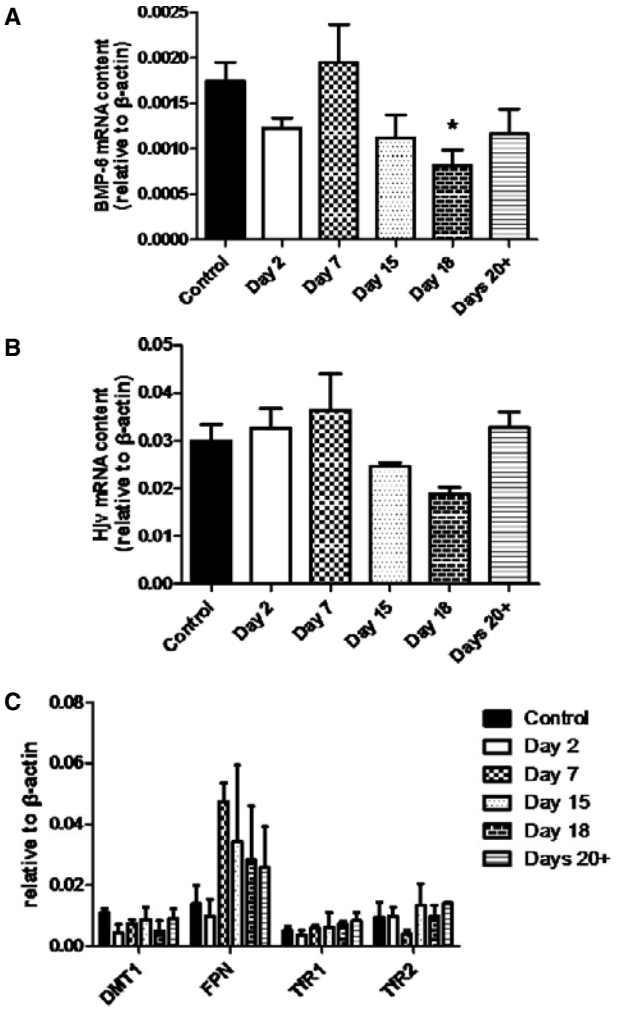

Fig. 4. Changes in mRNA levels of BMP-6 – bone-morphogenetic protein 6 (A), Hjv – hemojuvelin (B) and DMT-1 – divalent metal transporter 1, FPN – ferroportin, TfR1, TfR2 – transferrin receptor 1 and 2 (C) in the liver of mice with implanted tumours at different intervals after tumour implantation. *) significant in comparison with the control group.

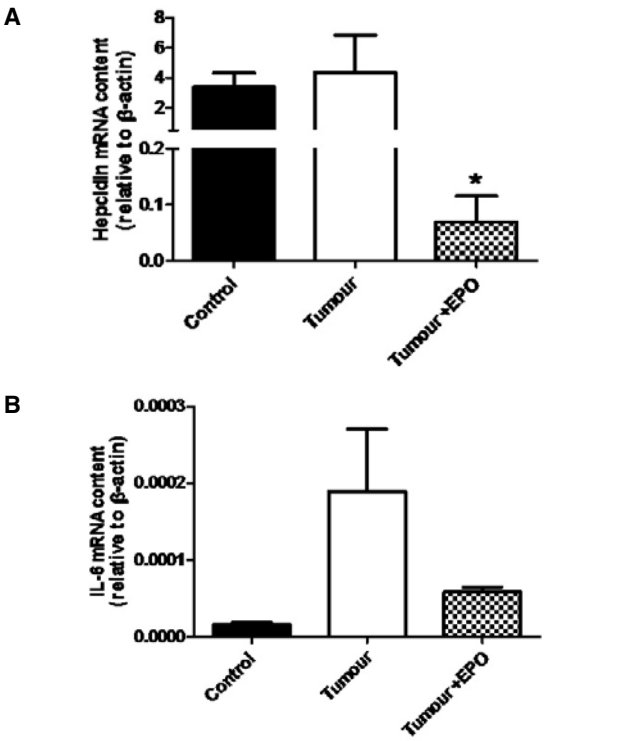

Fig. 5. Expression of hepcidin (A) and interleukin 6 (B) mRNA in the liver of mice with implanted tumours after a 4-day administration of erythropoietin (measured on day 22) – *) statistically significant in comparison to the control group and the group with implanted tumour without EPO administration (*$P < 0.05$).

the tumours of large size were strongly necrotic. However, in a parallel study we did not detect any important amount of mRNA for hepcidin in tumour tissue.

In late stages with significant tumour size and increased mRNA for IL-6, hepcidin expression increased despite persisting anaemia. However, the elevated hepcidin levels could still be decreased by erythropoietin administration. This was probably due to the enhanced erythropoiesis and its inhibiting effect on hepcidin expression despite the inflammatory hepcidin stimulation.

This is of importance as EPO is often administered to cancer patients (Dicato et al., 2010) and the relationship between erythropoietin, inflammation and hepcidin has substantial clinical consequences (Zucker, 2011). EPO is known to strongly decrease hepcidin expression (Nicolas et al., 2002b) but only when erythropoiesis is functional (Pak et al., 2006; Vokurka et al., 2006).

The results obtained in our model are in agreement with several observations. Erythropoietin-stimulated erythropoiesis can down-regulate hepcidin expression even in the presence of inflammation as explained on the level of STAT3 and Smad4 (Huang et al., 2009), and in fact it can be more powerful than inflammation (Theurl et al., 2009). Cancer patients with lower hepcidin concentration responded better to EPO treatment, and hepcidin can thus serve as a predictor of the sensitivity of EPO therapy (Katodritou et al., 2009a; Ukarma et al., 2009).

In contrast to other species including man, in mice two peptides – hepcidin 1 and hepcidin 2 – exist (Nicolas et al., 2001; Pigeon et al., 2001). Hepcidin 2 shares important homology with its analogue, but its overproduction has no influence on the phenotype (Lou et al., 2004). Its expression reacts to dietary iron (Mazur et al., 2003), to iron overload and EPO treatment, though it does not increase in response to lipopolysaccharide (Krijt et al., 2004). In agreement with this, hepcidin 2 expression in our experiments decreased in the same way as hepcidin; however, in late stages the expression of hepcidin 2 remained low in contrast to hepcidin, which increased in parallel with IL-6. Detailed knowledge of hepcidin 2 regulation remains elusive, but the measurement of the expression of both hepcidins in mice can be useful in differentiating the impact of iron metabolism and inflammation.

The expression of other genes showed rather limited and not significant changes. Only BMP-6 was significantly decreased on day 18 as well as hepcidin. This is

in agreement with the described decrease of BMP-6 and hepcidin in the liver of mice fed an iron-deficient diet (Kautz et al., 2008).

Anaemia in cancer has a complex pathogenesis and also its diagnosis and treatment require a complex attitude (Sasu et al., 2010). Several studies on the role of hepcidin in cancer have been published recently. Serum hepcidin was increased in patients with metastatic renal cell carcinoma and was correlated to IL-6 (Kamai et al., 2009). The role of IL-6 and hepcidin was proposed (Cucuianu et al., 2006) and reported in multiple myeloma (Sharma et al., 2008; Katodritou et al., 2009b). Hepcidin is up-regulated in both IL-6-dependent and -independent manner and may contribute to anaemia in these patients. Similarly, hepcidin contributed to anaemia of Hodgkin's lymphoma and inversely correlated with haemoglobin concentration in anaemic patients (Hohaus et al., 2010). On the other hand, systemic hepcidin was not supposed to be the cause of anaemia associated with colorectal cancer (Ward et al., 2008). Prohepcidin concentration did not seem to be correlated with haematological and iron parameters in a study of patients with solid tumours (Jacober et al., 2007). In general, hepcidin is supposed to be an effective marker for anaemia while prohepcidin is not (Sasu et al., 2010). More studies will be needed and in fact can be expected with the growing number of hepcidin assays available for routine clinical use.

Hepcidin was also studied in tumours and its possible role in cancerogenesis was argued. Hepcidin was down-regulated in the tumorous tissue of hepatocellular carcinoma (Kijima et al., 2008; Tan et al., 2009; Tseng et al., 2009), while it was expressed in colorectal carcinoma (Ward et al., 2008). It is not clear whether hepcidin of tumour origin can influence its systemic concentration, but it may represent a novel oncogenic signalling mechanism.

The implantation of tumour in our model does not correspond exactly to the natural development of human cancer, where the interaction with the immune system is more complex. However, it can simulate the different processes during the tumour growth. Rapidly growing tumour can consume iron and then be "visible" through the decrease of hepcidin, while the increase of hepcidin and IL-6 at later stages could serve as another marker of the body's reaction to tumour, especially when the hepcidin increase occurs in the presence of anaemia and iron deficiency. It can therefore contribute to ACD as observed in the clinical studies; nevertheless, we suppose that repeated measurements of hepcidin and its dynamics together with correlation to other parameters are necessary for its correct interpretation.

In conclusion, hepcidin liver expression in mice with implanted tumours in our experiments reacted to tumour growth, anaemia development and lack of iron, erythropoietin administration and inflammation, and it probably may contribute to the pathogenesis of anaemia in cancer. Its precise role requires further experimental and clinical investigation, as its expression in cancer probably varies and depends on complex clinical factors, such as the type of the tumour, the stage of the tumour, the immune response, inflammation, treatment and many others. We suppose that hepcidin measurement could become a useful tool in diagnostic procedures in cancer-associated anaemia and in the design of its therapeutic strategy.

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