博士論文要旨

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Induction and differentiation of hepatic cells from bone marrow cells and c-Met function

Introduction

Recent studies have shown that in vivo transplantation of mouse or rat bone marrow (BM) cells develop into hepatocytes. Furthermore, in in vitro culture, hepatocyte growth factor (HGF) has been identified as a factor for efficiently inducing differentiation of rat BM cells into hepatocyte-like cells.These findings have led to studies of hepatocyte regeneration from bone marrow stem cells for humans. In canines, there are many cases of severe hepatic disease for which effective treatment has not been established. In canine medicine, regenerative medicine will be an important treatment tool. However, for liver regeneration, the functions of HGF and c-Met, which is a specific HGF receptor, are important, but there are few studies regarding them in canine medicine.

The c-Met proto-oncogene is a receptor for HGF. Activation of the HGF/c-Met signal pathway leads to cell proliferation, motility, regeneration, and morphogenesis, so the signal pathway plays important roles in proliferation of malignancy or cell differentiation and proliferation. It has been suggest that HGF has a multiplicity of functions because of its expression profiles in various tissues and various physiological states. The overexpression of HGF or c-Met has been observed in the tumor cells of carcinomas and hematologic tumors such as hepatocyte carcinoma, lung cell carcinoma, and osteosarcoma. In the regenerating liver of mice and humans, c-Met expression increases and then plays an important role in liver regeneration. In canines, progressive investigations regarding HGF/c-Met are necessary to apply regenerative medicine, however, cloning and tissue distribution of canine c-Met gene have not been reported.

In Chapter 2, canine c-Met gene cloning and its tissue distribution are described. To investigate the function of c-Met in canine liver tissue regeneration, c-Met mRNA expression level in the liver was measured before and after partial hepatectomy.

In Chapter 3, as one function of HGF, it has been obvious that there is a relationship between HGF and some malignancies, such as hepatocyte carcinoma and lung cell carcinoma. However, the function of HGF in hematologic malignancies is largely unclear. Since canine hematologic malignancies resemble those in humans, studies of canine hematologic malignancies will contribute to both canine and human medicine. Also, investigation of the HGF/c-Met signal pathway in hematologic malignancies is necessary for the use of HGF in regenerative medicine. Based on this information, to determine the effects of HGF/c-Met in canine hematologic malignancies, c-Met mRNA expression was measured and compared with each disease category.

In Chapter 4, induction and differentiation of hepatocytes from canine bone marrow cells were performed. In this study, differentiation of hepatocytes from BM was examined by culturing BM cells by adding feline recombinant HGF and human placenta extract (Laennec). At the moment, from experiments using rats or mice, the technology to induce hepatocytes from BM is still inefficient, which is one of the issues making regenerative medicine impractical.

[Chapter 2]

Molecular cloning of canine c-Met and its expression after partial hepatectomy

To determine HGF functions in canine tissues including liver, it is necessary to clone canine c-Met cDNA and elucidate the mRNA expression profile. The c-Met proto-oncogene has been identified as the receptor for HGF. This molecule is a member of the cell surface receptor tyrosine kinase family and is a heterodimeric protein composed of an extracellular α-chain and a β-chain that spans the plasma membrane and includes an extracellular, a transmembrane and a cytoplasmic domain. cDNA has been cloned in a variety of species including human, murine, avian, and amphibian. In this chapter, the cloning and tissue distribution of canine c-Met are described. To investigate the effect of c-Met in canine liver regeneration, the level of c-Met mRNA expression in the liver was determined before and after partial hepatectomy.

RT-PCR was performed using total RNA extracted from canine liver and c-Met specific primers based on the sequences of human, rat and mouse cDNA, and DNA sequencing was performed on the Applied Biosystem Model 310 sequencer. The obtained sequence spanned 4419 bp and contained an open reading frame encoding a protein of 1383 amino acids. Canine c-Met amino acid showed high identity with human (89%), mouse (85%), rat (87%), chicken (68%), and xenopus (80%). c-Met mRNA was expressed in a variety of canine tissues including peripheral blood mononuclear cells (PBMC), bone marrow, liver, kidney, lung, stomach, uterus, testis, thymus, lymph node, small intestine, colon, adrenal gland, thyroid gland, heart, muscle, skin, pancreas, ovary, prostate, spleen, fat, cerebrum, and cerebellum. In addition, c-Met mRNA expression was examined in the liver before and after hepatectomy. The levels of the mRNA increased 1.986-fold in the liver after hepatectomy compared to that found in the liver before hepatectomy, indicating that c-Met is involved in various functions including remodeling of canine hepatocytes.

[Chapter 3]

c-Met expressions in canine hematologic malignancies

HGF/c-Met plays important roles in various malignancies. Overexpression of HGF in some cases of human myeloblastic and lymphoblastic leukemias and lymphomas has been determined. In some patients, high HGF production has unfavorable biological effects. However, little is known about the role of c-Met in hematologic malignancies, thus it is important to investigate c-Met effects: the only HGF receptor in hematologic malignancies. In the canine however, there is a high incidence of lymphoma and leukemia, similar to that in humans, and the pathophysiologies are still unclear.

In this study, to investigate the role of c-Met in canine hematologic tumors, bone marrow samples were collected from animals with acute myeloid leukemia, AML (1 case); chronic myeloid leukemia, CML (2 cases); myelodysplastic syndromes, MDS (5 cases); chronic lymphocytic leukemia, CLL (3 cases); and plasmacytoma (1 case). Mononuclear cells from peripheral blood were collected from one animal with acute lymphoblastic leukemia, ALL, (1 case). B lymphoma cell line and T lymphoma cell line were also used. In hematologic tumors, c-Met mRNA was increased compared to that of lymphoblastic leukemia. In MDS cases, c-Met amounts tended to be low compared to those of myeloblastic leukemia. c-Met mRNA expressions were lower in animals with lymphoblastic leukemia and MDS than in normal dogs.

[Chapter 4]

Induction and differentiation of hepatic cells from bone marrow cells using HGF or human placental extract

Recent studies demonstrate that in vivo transplantation of bone marrow cells (BM) differentiate into hepatocytes. Furthermore, HGF has been identified as a factor that effectively induces differentiation of BM cells into hepatocyte-like cells. Because BM collection is less invasive and the proliferative mechanism of BM is well understood, it could be a most promising tissue for regenerative medicine.

In this study, hepatocytes induced from BM by culturing BM with feline recombinant HGF or human placenta extract (Laennec) were examined. Laennec was used since it is known to promote

hepatocyte regeneration.

To assess the differentiation of BM cells into hepatocyte-like cells by adding HGF or human placental extract, we estimated the morphologic alteration of the cultured cells by adding each factor to BM cells. Albumin mRNA and proteins such as albumin, and protein components such as cytokeratin 8 (CK8), and cytokeratin 18 (CK18) as hepatocyte specific markers were detected. Furthermore, to examine the participation of HGF/c-Met in differentiation of hepatocytes from BM cells, auto-phosphorylation of c-Met inhibited HGF signaling by using synthetic peptides which imitated c-Met amino acid sequences in the intracellular domain. By RT-PCR analysis, albumin mRNA was first detected on day 14 in cells cultured with human placental extract and on day 28 in cells altered with HGF. For 28 day cultured cells, production of albumin and expression of CK 18 were analyzed by immunocytochemistry; both proteins were detected in cells cultured with human placental extract and feline recombinant HGF.

From this research, it became evident that canine BM cells could differentiate into hepatocytes and that the HGF/c-Met signal pathway played an important role. Additionally, human placental extract had the ability to differentiate BM cells into hepatocyte-like cells. Interestingly, BM cells cultured with human placental extract containing little HGF expressed albumin mRNA much earlier than when cultured with HGF. This suggests that human placental extract contained a superior factor that enhances the differentiation of BM cells into hepatocytes. In this regard, it is possible that a factor other than HGF induced hepatocytes from BM cells. An inhibition trial of c-Met auto-phosphorylation by adding synthesized peptides resulted in complete inhibition of differentiation. There are indications that HGF is an important inducer of differentiation of canine BM into hepatocytes. In human placental extract, factors known to promote hepatocyte proliferation are contained, including a small amount of HGF (0.13 ng/ml), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), transforming growth factor (TNFα, IL6, reptin, and dehydroepiandrosterone (DHEA). It is suggested that human placental extract contains another factor or other factors that promote differentiation of BM cells into hepatocytes. It is important to analyze further the factors included in human placental extract.

[Chapter 5]
Conclusions

In this study, in Chapter 2, the complete canine c-Met mRNA code was determined, elucidating the high homology of c-Met among animals. c-Met was determined to be an important factor for liver regeneration. In Chapter 3, it was proposed that canine c-Met plays a role in some hematologic malignancies. Also it is necessary to pay attention to the potential tumorgenesis of HGF when applying HGF to regenerative medicine, especially in vivo. In Chapter 4, canine BM cells differentiated into hepatocytes by HGF and human placental extract. For the differentiation of BM cells, HGF was identified as an important factor, supported by other factors included in human placental extract suggested to support efficient HGF functioning. Further analysis of the factors in human placental extract will contribute to regenerative medicine. These results will be important not only for the veterinary clinical sciences but also for the medical sciences.