# HEPATOCYTE GROWTH FACTOR IN MINK LIVER IN CASES OF MYCOSES

**Anda Valdovska, Aleksandrs Jemeļjanovs*, Māra Pilmane****
Faculty of Veterinary Medicine, LUA, Latvia, Anda.Valdovska@llu.lv
*Research Institute of Biotechnology and Veterinary Medicine „Sigra", LUA, Latvia
**Institute of Anatomy and Antropology, Faculty of Medicine, Riga Stradins University, Latvia

## ABSTRACT

In the present work we have studied the prevalence of fungal flora in the liver of 18 dark brown minks. We isolated 12 genus of moulds. Members of the genus *Candida, Aspergillus* and *Penicillium* were the most frequently isolated strains.

When mink are infected with fungi, the cell recruitment is impaired at the site of the infection, the tissue reaction is highly disorganized in target organs and the infection´s evolution is more severe. We studied HGF expression by immunohistochemistry.

Our results demonstrated that the distribution of hyphae were between hepatocytes in all areas of liver acinus, but fungal blastospores - mainly in interlobular bile ducts. Allocation of HGF in hepatocytes was observed uniform or non uniform, frequently around bile-ducts and portal tracts.

In conclusion, detection of HGF simultaneously to inflammation liver might be connected to the tissue ischemia and damage realized by the same inflammatory cells.
**KEY WORDS** mink, liver, mycoses, hepatocyte growth factor (HGF)

## INTRODUCTION

At present animal diseases such as mycoses caused by fungi are considered one of the most essential urgent issues of veterinary medicine in the world (Кузнецов, 2001). All substances absorbed *via* guts, including microscopic fungi, get into the organism through *v. porta*, with support the other veins that collect blood from the digestive tract, spleen, urinary bladder and pancreas (Klatskin and Conn, 1993; Junqueira *et al*., 1998). The liver constitutes the first barrier in the control of hematogenous dissemination for microscopic fungi of intestinal origin.

Frequently the macroorganism is not able fully to eliminate the pathogenic agent. Macrophages and lymphocytes mainly realize immune reactions. Microscopic fungi can intracellulary persist in these cells and generate chronic inflammation (Kellers, 1991; McGee *et al*., 1992; Jubb *et al*., 1993; Klatskin and Conn, 1993). When mink are infected with fungi, the cell recruitment is impaired at the site of the infection and microscopic fungi can disseminate *via* blood vessels in the body.

The liver is able to regenerate after damage. Cytokine hepatocyte growth factor (HGF) that is a potent mitogen of hepatocytes, performs multiple activities after liver injury (Watanabe *et al*., 2003; Hironobu *et al*., 2006). HGF in normal tissue is present in the inactive single chain form, and it is converted into an active heterodimeric form exclusively in the injured tissues (Miyazawa *et al*., 1996). HGF molecules are distributed to the liver, spleen, kidney and adrenal glands (Zioncheck, 1994). Sinusoidal endothelial cells and Kupffers cells contain HGF in normal liver (Maher, 1993), but during liver regeneration HGF is mainly produced by Ito cells (Hironobu *et al*., 2006), as well as fibroblasts, epithelial and endothelial cells, hepatocytes and Kupffers cells (Maher, 1993). As a result HGF participates in angiogenesis, morfogenesis and decreases cell apoptosis (Funakoshi and Nakamura, 2003).

The present study addresses the issue of a possible correlation between dissemination of mycosis and expression of growth factor in mink liver.

## MATERIALS AND METHODS

***Animals and tissue preparation.***To defect the fungal effect on the mink body, 18 dark brown mink at an age of seven months without clinical signs of any disease were selected randomly. The mink were brought from a fur farm of Riga district. These mink were anaesthetized with 1 ml 1 % solution of ditilini (Jepsen et al., 1981). Each liver sample was collected in a sterile bag for mycological examination and fixed in 12 % formalin too, embedded in paraffin and used for periodic acid – Schiff (PAS) staining and immunohistochemistry.

***Mycological examination*.** Sabouraud's agar was used as a primary isolation medium for the fungal cultures from the mink liver specimens (Willard *et a*l., 1994). A small surface of the changed tissue was burned on a flame, and small pieces of tissue from the middle were cut out with sterile scissors. The tissue cuts were used for a stripe-like inoculation onto media (Спесивцева, 1964) or they were placed on the agar surface (4-5 small tissue pieces in size of 0,5 cm X 0,5 cm (Quinn *et al*., 1994). All mycological inoculates on Petri plates were incubated in a thermostat for 4 weeks at a temperature of +26 ºC (Quinn *et al*., 1994; Кузнецов, 2001). The microscopic identification of the isolated fungi was carried out according to conventional methods (Саркисов *и др*., 1953; Kwon-Chung and Bennett, 1992; Bridson, 1993; Larone, 1995; Саттон *и др*., 2001).

***PAS method*** is a punctual method for detection of microscopic fungi in tissues. PAS staining painted microscopic fungi as pinkish red elements (Quinn *et al*., 1994). Multiple 6 µm-thick sections of the paraffin-embedded mink livers were deparaffinized, rehydrated, processed with 0, 5 % $K_2SO_4$ solution, washed in distillate water and seated in Shiff reagent. Hematoxylin was used for the counterstain. After tissue dehydratation, processing with polystirol was carried out.

***Immunohistochemistry*.** Multiple 6 µm-thick sections of the paraffin-embedded mink livers were examined for immunohistochemistry. The primary antibodies utilized in immunohistochemistry were rabbit polyclonal antibodies specific for HGF (dilution 1:300; code AF294NA, R&D System, Germany). Prior to immunostaining, sections were deparaffinized and rehydrated. Sections were processed in microwave for 20 min in 4 % citrate buffer (pH 10), quenched for 10 min with 3 % $H_2O_2$ for blocking endogenous peroxidase activity, rinsed in phosphate-buffered saline (pH 7,4), pretreated with a nonimmune goat serum for 10 min for blocking of nonspecific antibody binding and then incubated for 2 h with the primary antibodies. Immunoreaction was visualized by the other antibodies – avidin-biotin (LSAB) immunoperoxidase method using an LSAB kit (code K1015, DakoCytomation, Denmark) and DAB (diaminobenzidine) solution (code K3468, Dako, Denmark) was used as chromogen, and hematoxylin was used as the counterstain.

***Statistical analysis*.** For quantitative analysis we used a counting of inflammation cells in three fields of vision. Semi-quantitative analysis was used to estimate proportions of immunopositive cells in liver (Pilmane *et al*., 1998).

## RESULTS AND DISCUSSION

Proverbial, that portal vena (*v.porta*) with support of *v.mesenterica superior, v.mesenterica inferior, v.lienalis, v.cystica, v.gastrica sinistra, v.gastrica dextra* un *v.pregastrica* collects blood from the digestive tract, spleen, bile-cyst and pancreas (Jubb *et al*., 1993). Thereby there is a possibility of penetration in liver of peroraly received microscopic fungi.

**Microscopic fungi in the liver.** Micological examination showed a broad contamination with microscopic fungi in mink liver. The occurrence of fungi genus was: *Candida* 32 %, *Aspergillus* 16 %, *Penicillium* 12 %, *Wangiella* 7 %, *Arthrographis, Aureobasidium, Chaetomium, Scedosporium* – each per 6 %, but *Sporothrix* 4 %, *Cladosporium* 3 % and *Emmonsia* 2 %. It proves the great durability of this species. Most of the established

microscopic fungi are described in literature as widely spread environmental contaminants (Hubalek *et al*., 1998; Ponton *et al*., 2000; Кузнецов, 2001).

Most frequently *Candida spp., Aspergillus spp.* and *Penicillium spp.* were detected. Sutton et al. (Саттон *и др*., 2001) also confirms that they are mostly disseminated infection agents.

Contamination of animal liver with *Aspergillus spp.* after alimentary infection is also confirmed by other researchers (Carter and Chengappa, 1993; Quinn *et al*., 1994; Myrvik and Weiser, 1988; Carter and Wise, 2004). However, printed sources do not contain information regarding *Aspergillus* distribution in mink viscera. Althought pathogenic properties of A*spergillus* genus are less clear (Müllbacher and Eichner, 1984), nevertheless we suggest, that ability of *Aspergillus* to cause proteases promotes its distribution in mink liver, because protease could either assist tissue penetration by the fungus or degrade some critical host defense factor (Kwon-Chung and Bennett, 1992).

Candidosis has no geographic limitation (Edwards, 1991) and *Candida* is a normal commensal of the gastrointestinal tract of various warm-blooded animals (Kwon-Chung and Bennett, 1992; Кузнецов, 2001). Our finding of *Candida spp.* in the liver supports data of other researchers, who report generalization of mycosis (Macsween *et al*., 1979; Quinn *et a*l., 1994). Substantially, the data suggest that the main place of invasion of deep candidosis is oesophagus and stomach (Eras *et al*., 1972). Possibly, oesophagus and stomach, constitute the entrance for infection with minks.

Our finding of *Arthrographis kalrae* in experimental mink liver confirms Kwon-Chung and Bennett (1992) studies, who report pathogenity of aforementioned microscopic fungi concerning mice. Possibly, dimorfisms (growth ability at 37 $^{o}$C to 45 $^{o}$C) of *Arthrographis kalrae* explain our results (Саттон *и др*., 2001).

In spite of the limited data regarding *Aureobasidium pullulans* pathogenity, Pritchard and Muir (1987) report about *Aureobasidium pullulans* generated mycotic dermatitis. Sutton et al. (Саттон *и др*., 2001) inform about invasive mycosis among humans with impaired immunity. The aforementioned reports suggest the possibility to isolate *Aureobasidium pullulans* from mink's liver. Similarly *Chaetomium spp.* show ability to induce skin mycosis, allergic reactions and systemic mycosis (Саттон *и др*., 2001). Our findings confirm the data of the above mentioned reports regarding *Chaetomium spp.* as a possible causative agent of systemic mycosis.

Microscopic fungi *Sporothrix cyanescens,* is one representative of *Sporotrichum,* which is widespread in the environment. Our finding of *Sporothrix cyanescens* is confirmed by other scientists, who isolated this yeastlike microscopic fungi from animal viscera (Кузнецов, 2001). Although animal mycosis sporotrihosis mainly is induced by *Sporothrix schenckii* (Фейер *и др*., 1966; Кузнецов, 2001). At the same time other author (Спесивцева, 1964) reported others species for *Sporotrichum,* which are able to penetrate into the organism *via* skin and mucous membrane wounds.

*Wangiella* microscopic fungi mainly induce subcutaneus mycosis of cats, dogs, horses, goats and other animals (Quinn *et al*., 1994). Our finding of *Wangiella spp.* are confirmed by other authors, who indicate the ability of aforementioned microscopic fungi to generate systemic mycosis (Quinn *et al*., 1994).

Dimorphism of *Emmonsia* is a possible reason for our findings of this microscopic fungi in mink liver (Hubalek *et al*., 1998). Larone (1995) testifed the ability of *Emmonsia spp.* to generate pulmonic inflammatory process in rodents. The listed data support Hubalek (1998), whose studies show distribution of *Emmonsia* thanks to small rodents, who are carriers of a pathogenic agent. Possibly, *Emmonsia spp.* penetrate into mink organism from the contaminated cage bedding.

Stanley *et al.* (1992) research data approved isolating of *Scedosporium prolificans* from viscera. Authors pointed out the reason for aforementioned pathogenity that it is growth ability until a temperature of 45 °C (Саттон *и др*., 2001).

Among all types of microscopic fungi found in mink liver there were 42 % of *Dematiaceous* group fungi (*Aureobasidium pullulans, Chaetomium spp., Cladosporium spp., Scedosporium spp., Wangiella spp.*) (Kwon-Chung and Bennett, 1992). The common feature of these fungi is the presence of melanin in the cell wall. Other researchers (Zhdanova and Vasilevskaya, 1990) have proved that the presence of melanin pigment affects essentially the endurance of these cells against the influence of environmental moisture, temperature and radiation of the sun as well as providing vitality in the surroundings with an insufficient amount of nutrients. In addition, when in a body, the dark pigmented microscopic fungi containing melanin have an increased endurance against the body immunity protective factors.

With specific staining method in the formalin-embedded mink livers we detected PAS - positive yeast-like fungi. Our findings in mink liver show distribution of hyphae between hepatocytes in all area of liver acinus, but fungal blastospores mainly in interlobular bile ducts and in small numbers between hepatocytes in periportal area closely to triadas and around central veins. We explain findings of microscopic fungi in perivenular area with an individuality of venous blood circulation in liver, because portal veina is divided in venous capillary, which flow together, establish liver veins (Junqueira *et al*., 1998).

Thereby pathologic agents from the stomach and gut can get into the perivenular area. Contamination with microscopic fungi of periportal area we explain with research data, who reported, that the necessity of oxygen for Y (yeastlike) forms is 5 – 6 time higher than M (micelial) forms of microscopic fungi. Our findings of yeastlike forms in bile ducts is confirmed by other scientists (Jubb *et al*., 1993), who report that mycotic pathogenic agents often contaminate bile ducts, while pathogenic agents can not be detected in blood circulation and in viscera.

Despite the large amount of mycologically detected microscopic fungi in mink liver, our research shows a small quantity of fungal blastospores and hyphae. Possibly, our results suggeste, that any staining of histological preparation does not provide uniform staining of all microscopic fungi in tissues. Relatively new cells of microscopic fungi, which contain a large amount of chromatin, are better stained with alkali contained stains, but older forms, which contain a small amount of chromatin, are better stained with acid containing stains (Фейер *и др*., 1966).

**Hepatocyte growth factor (HGF)** in normal hepatocytes persist in a non active form (Miyazawa *et a*l., 1996; Ishikawa *et al*., 2001). In case of liver damage or irritation, HGF is transformed in to active form and hepatocytes become immunohistochemically able to respond with anti-HGF antibody (Ljubimova *et al.*, 1997). Our research results show HGF expression in mink livers. We mainly observe allocation of HGF in hepatocytes, uniform or non uniform, frequently around bile ducts and portal tracts. Our results of HGF expression in cytoplasma of hepatocytes is supported by Thomson and Lotze (2003). Recognized dispersion of HGF in our research, mainly around bile ducts and portal tracts, fall within areas, where we mainly establish inflammation and the presence of yeastlike microscopic fungi, because HGF stimulates not only the proliferation of liver parenchyma, but also the proliferation of biliar epithelial cells (Joplin *et al*., 1992). HGF in liver can also be produced by non parenchymal cells, for example, hepatic stellate cells, Kupffer cells, sinusoidal endothelial cells (Maher, 1993). Thereby HGF operates as a factor, which promotes liver regeneration during damage (Ljubimova *et a*l., 1997; Ishikawa *et al*., 2001; Watanabe *et al*., 2003; Hironobu *et al*., 2006). Between non parenchymal cells HGF expression we suggest in endotheliocytes of veins, which, possibly approves compensatory activity of HGF during inflammation.

In summary, simultaneously to other authors, we conclude, that the greatest part of isolated microscopic fungi is widely distributed in the environment (Hubalek *et al*., 1998; 2000; Кузнецов, 2001) and might be incorporated into the animal body *via* damaged digestive tract. The last possibility is described also by Фейер *и др*. (1966), Кузнецов (2001) and Xi *et al.* (2004). In our animals the damaged digestive tract might be a result of sharp bone particles in ready-to eat feed used for minks. Detection of HGF simultaneously to inflammation liver might be connected to the tissue ischemia and damage realized by the same inflammatory cells.

**REFERENCES**


1. Bridson E. (1993) *The oxoid vade-mecum of microbiology*, Pub. by Unipath Ltd, Basingstoke, p.85 – 128
2. Carter G. R., Chengappa M. M. (1993) *Microbial diseases. A veterinarian's guide to laboratory diagnosis*, Iowa State Press, Ames, 293 p.
3. Edwards J.E., 1991 Invasive *Candida* infections. *N. Engl. J. Med*. 324: 1060 – 1062
4. Eras P., Goldstein M.J., Sherlock P. (1972) *Candida* infection of the gastrointestinal tract. *Medicine*, 51: p.367 – 379
5. Funakoshi H., NakamuraT. (2003) Hepatocyte growth factor: from diagnosis to clinical applications. *Clinica Chimica Acta*, 327: p.1 – 23
6. Hironobu M., Hiroaki S., Hiroshi I., Fumio K., Satoshi A., Akira T., Masayuki O., Hiroyuki Y., Atsushi K., Hideyuki Y., Shigeaki S., Masaru M. (2006) Changes in growth factor and cytokine expression in biliary obstructed rat liver and their relationship with delayed liver regeneration after partial hepatectomy. *World Journal of Gastroenterology*. 12 (13), p.2053-2059
7. Hubalek Z., Nesvadbova J., Halouzka J. (1998) Emmonsiosis of rodents in an agroecosystem. *Journal Medical mycology*. 36, p.387-390
8. Ishikawa K., Masui T., Ishikawa K., Shiojiri N. (2001) Immunolocalization of hepatocyte growth factor and its receptor (c-Met) during mouse liver development. *Histochem cell biol*, 116: p.453 – 462
9. Jepsen O.R., Poulsen F.S., Jorgensen G. (1981) Collection of blood, sedation and anaesthesia in mink. *Nordisk veterinaermedicin*, Suppl.1, p.9-13.
10. Joplin R., Hishida T., Tsubouchi H. (1992) Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. *The Journal Clinical of Investigation*. 90, p.1284-1289
11. Jubb K.V.F., Kennedy P.C., Palmer N. (1993) *Pathology of Domestic animals*, 4th ed., vol.2., Academic Press, London, p. 255 – 359
12. Junqueira L.C., Carneiro J., Kelley R.O. (1998) *Basic histology*. 9th ed., Prentice-Hall, p. 75 – 317.
13. Kellers R. (1991) *Ievads imunoloģijā un imūnpatoloģijā* (Introduction in imunology and imunopathology). Zvaigzne, Rīga, 28. – 261. lpp. (In Latvian)
14. Klatskin G., Conn H.O. (1993) *Histopathology of the liver*, Vol. 1, Oxford University Press, USA, p. 9 – 327.
15. Kwon-Chung K. J., Bennett J. E. (1992) *Medical mycology*. Lea & Febiger, Philadelphia, 826 p.
16. Larone D. H.. (1995) *Medically important fungi. A guide to identification*. Third ed., ASM Press, Vashington, 261 p.
17. Ljubimova J.Y., Petrovic L.M., Arkadopoulos N., Blanc P., Geller S.A., Demetriou A.A. (1997) Lack of hepatocyte growth factor receptor (c-met) gene expression in fulminant hepatic failure livers before transplantation. *Digestive Diseases and Sciences*, Vol.42, 8: p.1675 – 1680

18. Macsween R.N.M., Anthony P.P., Scheuer P.J. (1979) *Pathology of the liver*. Churchill Livingstone, New York, p. 23 - 205
19. Maher J.J. (1993) Cell specific Expression of Hepatocyte growth factor in liver. *Journal Clinical Investigation*. Vol. 91, p.2244 – 2252
20. McGee J.O'D., Isaacson P.G., Wright N.A. (1992) Oxford textbook of pathology. Vol. 1 *Principles of pathology*. Oxford University Press, USA, p.321-491.
21. Miyazawa K., Shimomura T., Kitamura N. (1996) Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. *The Journal of Biological Chemistry*, Vol.271, 7: p.3615 – 3618.
22. Müllbacher A., Eichner R.D, 1984 Immunosuppression *in vitro* by a metabolite of a humans pathogenic fungus. *Proc. Natl. Acad. Sci*., 81: p.3835 – 3837.
23. Myrvik Q.N., Weiser R.S. (1988) *Fundamentals of medical bacteriology and mycology*. 2 ed., Lea & Febiger, Philadelphia, p.501 - 555
24. Pilmane M., Rumba I., Sundler F., Luts A. (1998) Patterns of occurrence and distribution of neuroendocrine elements in lungs of humans with chronic lung diseasess. Proceedings of the Latvian Academy of Sciences. Vol.52, number 3/4 (596/597), p.144 – 152.
25. Quinn P.J., Carter M.E., Markey B.K., Carter G.R. (1994) *Clinical veterinary microbiology*, Wolfe Pub., London, p.368 – 375
26. Stanley M.W., Deike M., Knoedler J., Iber C. (1992) Pulmonary mycetomas in immunocompetent patients: diagnosis by fine – needle aspiration. *Diagn. Cytopathol.* 8, p.577-579
27. Thomson A.W., Lotze M.T. (2003) *The citokine handbook*. 4th ed., Vol. II, Academic Press, London, p. 792 – 1152
28. Zioncheck T.F., Richardson L., De Guzmann G.G., Modi N.B., Hansen S.E., Godowski P.J. (1994) The pharmacokinetics, tissue localization and metabolic processing of recombinant human hepatocyte growth factor after intravenous administration in rats. *Endocrinology*. 134, p.1879-1887
29. Zhdanova N.N., Vasilevskaya A.I. (1990) Taxonomic significance of certain chemototaxonomic characters in soil melanin-containing micromycetes. *Тезисы докладов I Всесоюзного совещания*, Киев, 16-18 мая 1990 г.,25-26.
30. Watanabe H., Sumi S., Kitamura Y., Nio Y., Higami T. (2003) Immunohistochemical Analysis of Vascular Endothelial Growth Factor and Hepatocyte Growth Factor, and Their Receptors, in Transplanted Islets in Rats. Journal Surgery Today, 33, p.854 -860
31. Willard M.D., Tvedten H., Turnwald G.H. (1994) *Small animal clinical diagnosis by laboratory methods*. 2nd ed., W.B.Saunders comp., Philadelphia, p. 56-57.
32. Фейер Э., Олах Д., Сатмари Ш., Содорай Л., Ури Й. (1966) *Медицинская микология* (Medical mycology). Akademiai Kiado, Budapest, pp.23-308 (In Russian)
33. Кузнецов А. Ф. (2001) *Ветеринарная микология* (Veterinary mycology). Лань, Санкт-Петербург, 410 p. (In Russian)
34. Саркисов А. Х., Капустин В. Ф., Квашнина Е. С., Королева В. П. (1953) *Атлас грибов патогенных для сельскохозяйственных животных и птиц* (Atlas of pathogenic fungi concerning agricultural animals and birds). Государственное издательство с-хозяйственной литературы, Москва, 158 с. (In Russian)
35. Саттон Д., Фотергилл А., Ринальди М. (2001) *Определитель патогенных и условно патогенных грибов* (Guid to clinically significant fungi). Мир, Москва, 454 с. (In Russian)
36. Спесивцева, Н. А. (1964) *Микозы и микотоксикозы* (Mycoses and mycotoxicoses). Колос, Москва, с. 3 – 473 (In Russian)