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Original article

Histopathologic and immunohistochemical lesions in liver of mink infected with Aleutian disease virus

A. Valdovska[1], M. Pilmane[2]

[1] Faculty of Veterinary Medicine, Latvian University of Agriculture, 8 K. Helmana street, Jelgava, LV – 3004, Latvia
[2] Institute of Anatomy and Antropology, Riga Stradins University, 16 Dzirciema street, Riga, LV 1007, Latvia

Abstract

Parvovirus of Aleutian disease causes mainly damage to kidneys, but immune complexes deposition and damage may occur also in other organs. In mink farms of Latvia the liver dystrophy or hepatic lipidosis of mink is widely distributed. The goal of this study was to examine probability of liver damage and regeneration of mink infected with Aleutian disease virus. Liver injury was assessed histologically. The mink liver demonstrated inflammation of liver parenchyma and foci of fatty liver. In immunohistochemistry, during liver regeneration the matrix metalloproteinases MMP-9, vascular endothelial growth factor and β-defensin 2 expressions were lower, but MMP-2 and nerve growth factor receptor p75 expression was increased.

Key words: liver, mink, Aleutian disease, immunohistochemistry

Introduction

Aleutian disease (AD) is a most important, chronic, progressive, non-treatable disease of mink caused by a parvovirus. Histological lesions are present in a variety of organs, but most relevant lesions mainly are observed in the kidneys (Hunter 1996).

Liver lesions reported in mink with AD include mononuclear cell infiltration in portal areas of the liver, bile ducts proliferation, periportal fibrosis and individual hepatocyte necrosis. The classic or chronic form of AD is not caused by direct viral damage of tissues, but rather by immune response resulting in the formation of immune complexes and their subsequent deposition in glomeruli of the kidney, arterial walls and other organs. Commonly, vascular lesions occur mostly in small to medium-sized arterioles where they are formed as the result of degeneration of the blood vessels (Hunter 1996).

Liver is the major metabolic center of the animal and this organ is able to regenerate after damage. Because the host response to viral antigen ultimately leads to the death of the mink, still a question remains open on the processes of degeneration and regeneration in liver and it is important to expand our understanding of liver histopathological analysis of mink infected with Aleutian disease virus.

Correspondence to: A. Valdovska, e-mail: Anda.Valdovska@llu.lv, tel.: 371 26525501, fax: 371 63027344

Materials and Methods

Animals and tissue preparation. To detect the histopathological changes in liver, clinical healthy 18 dark brown minks of seven months of age were randomly selected. All minks were positive to virus of AD according to reaction of imunoelectroosmophoresis. The mink were brought from a fur farm of Riga district. After parenteral euthanasia of minks with 1 ml 1% solution of ditilini (Jepsen et al. 1981) their liver samples were fixed in 12% formalin. All experimental minks were obtained within the period of pelting and slaughtering and in accordance to guidelines of animal protection.

Immunohistochemistry. Multiple 6 µm-thick sections of the paraffin-embedded mink liver were examined for immunohistochemistry (Hsu et al. 1981). 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 to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (pH 7.4), pretreated with a nonimmune goat serum for 10 min to block a nonspecific antibody binding and then incubated for 2 h with the primary antibodies.

The primary antibodies utilized in immunohistochemistry were rabbit polyclonal antibodies specific for hepatocyte growth factor (HGF, dilution 1:300, R&D System, DE), nerve growth factor receptor p75 (NGFR p75, dilution 1:150, DakoCytomation, DK), vascular endothelial growth factor (VEGF, dilution 1:50; DakoCytomation, DK), matrix metalloproteinases MMP-2 (dilution 1:100, R&D System, DE) and MMP-9 (dilution 1:100, R&D System, DE), fibroblast growth factor receptor 1 (FGFR 1, dilution 1:100, Abcam, UK), fibronectin (Fn, dilution 1:100, Dako, DK), laminin (Lm, dilution 1:25, Euro-Diagnostica, DK), type IV collagen (dilution 1:25, Euro-Diagnostica, DK), and β-defensin 2 (dilution 1:100, R&D System, DE).

Immunoreaction was visualized by the avidin-biotin (LSAB) immunoperoxidase method using an LSAB kit (DakoCytomation, DK), and DAB (diaminobenzidine) solution (Dako, DK) was used as chromogen, and hematoxylin was used as the counterstain.

TUNEL reaction was used for detection of apoptosis (Negoescu et al. 1998). *In situ Cell Death Detection*, POD (Roche Diagnostics) and DAB substrate (Vector) were used. Deparafinised sections (xylol 2 x 4 min, 99% ethanol 2 x 2 min, 95% ethanol 2 x 2 min and 70% ethanol 2 x 2 min) were rinsed with water (7-10 min) and transferred to PBS (pH 7.5) for 10 min. Subsequently slides placed into 50 ml PBS solution with 500 µl 30% hydrogen peroxide for 30 min on shaker to block the endogenous peroxidases. Afterwards tissue samples were washed with PBS (3 x 5 min), placed into 0.2 M boric acid (pH 7.0), placed into microwave (700 W) for 10 min for fixation of antigen, cooled to room temperature and rinsed with PBS. After that, slides were kept in refrigerator in 0.1% BSA (bovine serum albumin) solution with PBS for 10 min and then incubated in TUNEL mix (Tdt – mix of terminal deoxynucleotide transferase and DIG-labeled deoxynucleotide) for 1h at +37°C. Then the slides were rinsed with PBS 1:10, and incubated for 30 min at +37°C with POD (sheep anti-digoxygenin antibogy coupled with horseradish peroxidase Fab fragment). Then the slides were washed with PBS, covered with DAB (diaminobenzidine chromogen) for 7 min, and then rinsed with running water for 5 min. Finally, haematoxylin and eosin staining was performed on each sample. Sections were covered with a polystyrene-based medium and coverslipped.

Statistical analysis. For quantitative analysis we used a counting of inflammation cells in three fields of vision, while semi-quantitative analysis was used to estimate proportions of immunopositive cells in liver (Pilmane et al. 1995). The designations were as follows: 1 – few positive cells in the view field; 2 – moderate and 3 – numerous positive cells in the view field.

Results

In the samples of mink liver the lobular architecture has been maintained, but inflammation infiltrate, being rich in lymphocytes, was extending from the portal tracts towards side parenchyma. In all histological samples of liver, macrophages constituted 18%, neutrophil leukocytes – 27%, but lymphocytes – 55% of the total inflammation cell count. A strongly marked tissue infiltration with macrophages, neutrophil leukocytes and lymphocytes were found around the central vein of lobuli, but Kupffer cells infiltration – mainly around portal tract ducts.

The analysis of the tissue sections stained for FGFR1 showed that the receptor immunoreactivity was observed in lymphocytes and macrophages. In periportal area, only moderately positive cells were detected. It was interesting to find out that FGFR1 expression is directly associated with the proliferation of inflammation cells (Fig. 1), furthermore, this impact to increase of all cell types was significant.

The increment of VEGF (moderately positive cells) activity was basically observed in portal hepatocytes. The factor has been found also in liver blood vessels. Research does not suggest a positive relation between inflammation cells and of VEGF expression, but the coherence in mink liver was observed – if there were only few apoptotic hepatocytes, then VEGF staining was significant.

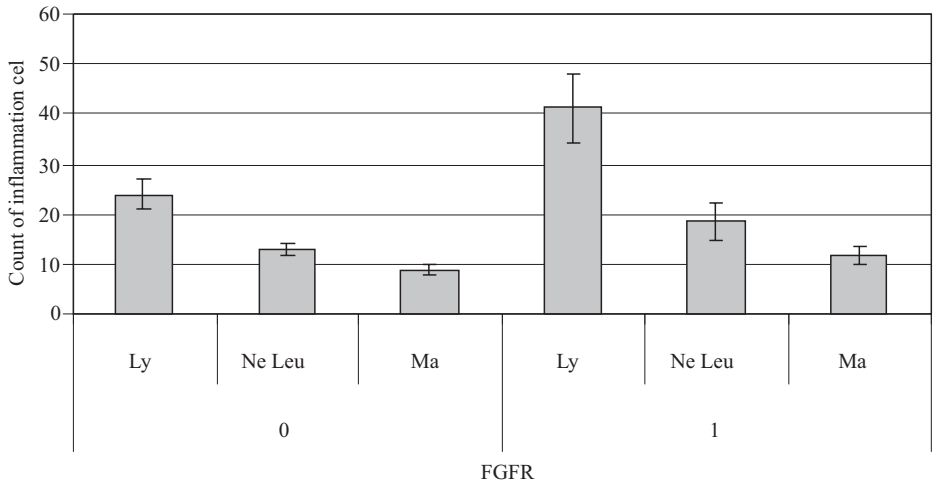

Fig. 1. Relation between number of inflammatory cells and FGFR

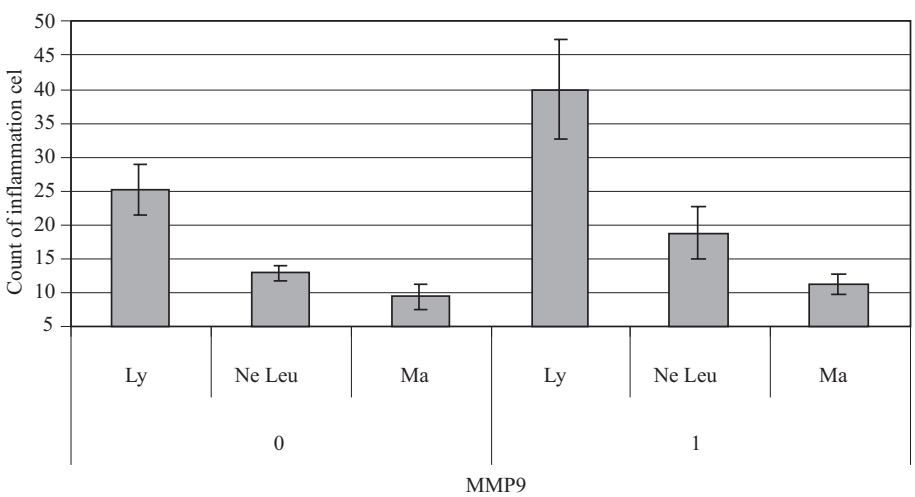

Fig. 2. Relation between MMP-9 containing structures and inflammatory cells
Ly – lymphocytes
Ne Leu – neutrophil leukocytes
Ma – macrophages
FGFR – fibroblast growth factor receptor
MMP9 – matrix metalloproteinases 9

Numerous cells positive for NGFR p75 of hepatic stellate cells (HSC) were demonstrated, mainly around bile ducts and in periportal area. We also observed a relationship – moderate hepatocyte apoptosis correlated to the more intensive expresion of NGFR p75, at the same time if there were numerous apoptotic hepatocytes, NGFR immunoreactivity was not seen.

Matrix metalloproteinase MMP-2 (gelatinase A) in liver lobules was discharged by macrophages and occasionally by fibroblasts. The correlation between MMP-2 and inflammation apparent intensity was absent despite numerous inflammatory cells observed in parenchyma. MMP-9 (gelatinase B) was seen in Kupffer cells and occasionally in lymphocytes. Structures positive to MMP-9 were often clustered close to blood vessels. Statistically a confirmation was obtained (Fig. 2) that changes of inflammation cell count correlated with the number of cells containing MMP-9. The difference between the types of increased inflammatory cells was significant. Consequently, gelatinases B-positive structures in tissues correlated directly with the inflammation process.

β-defensin 2-stained hepatocytes were mainly scattered across portal tracts and expression of moderate or numerous positive cells in all investigated specimens was seen. A relation (Fig. 3) between inflammation cells and defensin expression was detected – with increasing of lymphocytes and leukocytes the increase in expression of defensin was observed.

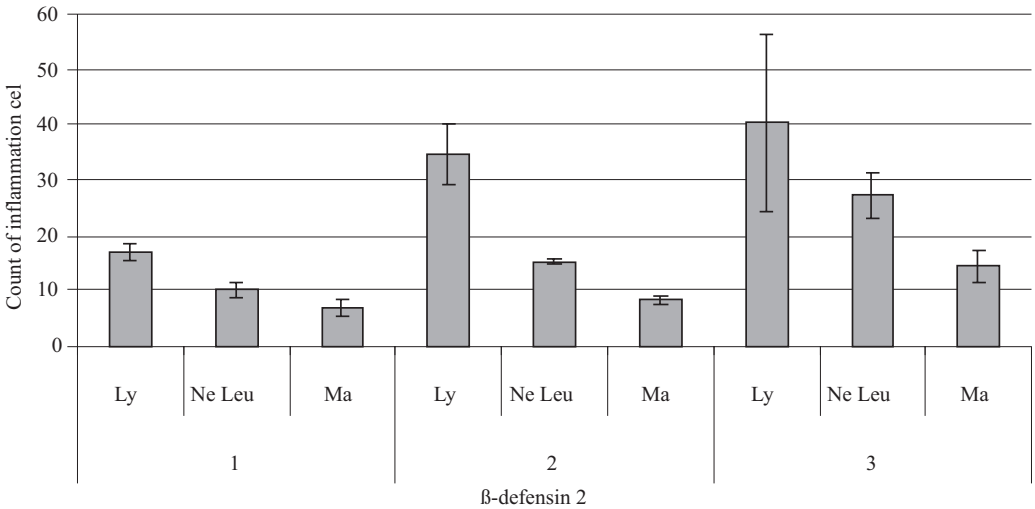

Fig. 3. Relation between inflammation cells and β-defensin 2 containing structures

Expression of HGF in hepatocytes was observed focally around bile ducts and portal tracts. The weak staining for the above-said factor was seen also in endoteliocytes of blood vessels (interlobular vein). The expression of HGF was found in the zones, where lots of the inflammation cells were observed. Upon assessing statistically a correlation between inflammation cells and apoptosis of hepatocytes, it turned out that when apoptosis was very extensive or average, there was no considerable difference in expression of inflammation cells whereas when apoptosis was less extensive – their number was considerably lower.

Fragmental laminin, type IV collagen and fibronectin immunoreactivity were detected in basal membrane of blood vessels of mink liver. However, basal membrane of bile ducts didn't show any immunoreactivity to the above-mentioned components. Components of basal membrane in mink liver were practically absent in the case of moderate expression of MMP.

Apoptosis of hepatocytes in liver samples of all animals and in all parts of parenchyma was detected. Interestingly, it turned out that when a moderate or numerous apoptotic hepatocytes were seen, there was no considerable difference in expression of inflammation cells, whereas when just a few apoptotic cells were present – their number is considerably lower. The statistical analysis showed that the influence of inflammation cells on apoptosis is not significant.

Discussion

At necropsy liver removed from affected mink displayed most frequently signs of hepatitis without others lesions. In liver samples the lobular architecture had been maintained, but a strongly marked tissue infiltration with inflammation cells, that was found around the central vein and portal tracts (triads) of liver, showed that there was a pathological process. The presence of neutrophil leukocytes in tissues is a proof of an acute inflammatory process. Klatskin and Conn (1993) confirmed that a neutrophil infiltration of the liver is observed in case of hepatitis and its placement around the triad is an abnormal phenomenon. Inflammation is a complex reaction and usually only some lymphocytes can be found around the triad in comparison with our finding and authors characterize such a picture as a chronic active hepatitis inclined to liver necrosis. Although Kupffer cells are most often found in the first section of acinus (Kelly 1993) still we found this cell infiltration in liver samples not only around portal ducts but also around the central vein. Infiltration of Kupffer cells develops on later inflammation stages and characterizes intensity of immunity and in case of a chronic inflammation, macrophages become active in the inflamed tissues.

Kupffer cells are relevant to the protection of an organism (Laskin 1990), but activated macrophages must initiate activation of liver HSC that can cause liver fibrosis and apoptosis of hepatocytes (Oakley et al. 2003, Friedman 2005). In case of fibrous damage, hepatocytes express NGF (or neurotropine) (Oakley et al. 2003), but NGF activity is also increased by NGFR p75 from activated liver HSC (Trim et al. 2000, Wu and Zern 2000, Passino et al. 2007). Observation that an average hepatic stellate cells count correlated with apoptosis of hepatocytes indicates a response of liver to the damage. Researchers have also found that a major trait of a regeneration process is apoptosis of activated HSC (Issa et al. 2001). We concluded that the NGFR p75 is discharged from hepatocytes during liver damage and it is connected with apoptosis of HSC, however, NGFR expression is not vital during liver apoptosis (Oakley et al. 2003), which

possibly is HSC adaptive and compensatory response not only to apoptosis itself but also to inflammation and/or dystrophy that is testified by NGFR expression more around bile ducts and in periportal area.

A question still remains open on the course of inflammation process where one of the characteristic features is FGFR activity. Our findings of FGFR in lymphocytes and macrophages coincide with the data on the fact that a high and low activity FGF receptors are present in cells and a complex FGF/FGFR is placed in the cell nucleus (Ensoli et al. 2003). As FGFR is involved in extracellular matrix (ECM) degradation control, FGFR expression level in directly influences cell proliferation and migration, therefore our research results are indicative of a proliferative process of inflammation cells, furthermore, of a chronic course of the process. FGFR expression is observed basically in periportal part, still it characterizes a chronic acute inflammation.

Vascular endothelial growth factor, which is also known as vascular permeability factor – is one of the strongest endothelial penetrability factors (Essser et al. 1998, Rajkumar 2001, Nolan et al. 2004). VEGF promotes vascular disruption, increases vascular permeability and contributes to inflammation process (Taniguchi et al. 2001). Also Aleutian disease virus and mediators of inflammation induced damage to blood vessels and increased its permeability. The increment of VEGF activity was found basically in portal hepatocytes, althought VEGF can also be produced by others cell types – macrophages, blood platelets, leukocytes, polimorphonuclear neutrophils (Coenjaerts et al. 2004). Consequently, the factor has shown higher activity in liver sections, which are richly supplied with oxygen. Hypoxia is stimulating VEGF expression. VEGF has also been found in liver blood vessels what indicates the decomposition and increased permeability of blood vessels, as a result the inflammation process develops (Essser et al. 1998, Taniguchi et al. 2001). Regardless of the fact that VEGF is produced by several types of cells, including macrophages, thrombocytes, leukocytes, polymorphonuclear neutrophils, within our research no positive correlation has been confirmed between inflammation cells and VEGF expression intensity. This proves that a factor with a VEGF-blocking effect is present in mink organism. It turns out that a virus of genus *Parvoviridae* (consequently Aleutian virus also) has an oncosuppressive effect and blocks VEGF activity (Blechacz and Russel 2004). The observed correlation – if there are only some apoptotic hepatocytes then VEGF distribution is a considerable one, shows that as the result of VEFG increment the response of the said organ to the damage is facilitated and it is connected with maintenance of sinusoidal homeostasis during liver dystrophy. In general, the increase of VEGF activity in periportal hepatocytes in animals with the commenced apoptosis points to a possible antiapoptotic role of this growth factor, that agrees with other authors reports (Ueda et al. 2006).

Matrix metalloproteinases are endopeptidases, activity of which is regulated by inflammation and immunologic processes (Hanumegowda et al. 2003, Friedman 2003, Visse and Nagase 2003). The main function of MMP is degradation of extracellular matrix (Hanumegowda et al. 2003) as a result the exchange of gases and substances between blood and hepatocytes is considerably mitigated (Kelly 1993, Klatskin and Conn 1993). The lack of correlation of MMP-2 with any groups of inflammation intensity observed by us could be explained by the fact that although neutrophilic leukocytes are a significant source of reactive oxygen species (Thannickal and Fanburg 2000), still an activated neutrohil produces also oxygen monoxide, which in an opposite case reduces the initial discharging of peroxide in cells blocking MMP-2 secretion and reducing formation of fibrosis in liver (Asai 2006). Arthur (1998) has also made a similar observation, stating that there was little gelatinase A in a healthy liver but it dramatically increased in case of liver diseases. However he made a conclusion that as fibrosis is progressing, MMP-2 activity either does not exist or it is very marginal. MMP-9 finding in Kupffer cells and a small amount of it also in inflammation cells in blood vessels could be explained by the activity of Kupffer cells from which infiltration is developing on later inflammation stages and in case of the chronic inflammation they get activated in the inflamed cells. Structures positive to MMP-9 often were clustering close to blood vessels, which coincides with observations of other scientists, as activated collagenases are localizing near the basement membrane and affect it colagenolytically (Hanumegowda et al. 2003). It was detected that variations of inflammation cell count are correlating with number of cells containing MMP-9, moreover this correlation is significant. Consequently, the level of gelatinases B (MMP-9) in tissues correlates directly with the inflammation process, which agrees with conclusions made by other researchers (Reif et al. 2005).

A major function of matrix metalloproteinases is degradation of extracellular matric components (Arthur 1997, Friedman 1999, Hanumegowda et al. 2003) however collagen type IV and laminin are also present in the basement membrane (Frappier 2006), including blood vessels. The founding of fragmental basement membrane components in mink liver indicates an increasing permeability of the basal membrane and a reduced preventive barrier in blood vessels wall. Thus, it induces reduced protective conditions (Brown et al. 2006). Components of the basement membrane in liver of experimental minks were practically absent in the case of marked expression of matrix metalloproteinases that proves the presence of a significant

inflammation process. This is confirmed by the finding that inflammation cells, particularly macrophages, are able of an active synthesis and discharging of MMP-2 and MMP-9 of a type IV collagenases (Birkedal-Hansen 1993, Hanumegowda et al. 2003), which lyse the components of the basement membrane, a type IV collagen and laminin as well as proteoglycans, gelatins, elastyn and fibronectine (Twinning 1994). A likely influence of metalloproteinases on the components of the basement membrane is suggested by the fact that structures positive to MMP-9 in experimental mink liver were often placed close to blood vessels. Virus of Aleutian disease is also a significant factor of the damage of blood vessel walls. Pathogenesis of disease is based on ability of the virus to form complexes in blood (virus-antibody-complement), which attach themselves to the walls of blood vessels, forming clusters and causing arteritis as well as a disappearance of the basement membrane.

Defensins are one of the major families of antimicrobial peptides (Lehrer et al. 2002). They were shown to be effective against HIV and other viruses (Smet and Contreras 2005). It is noted that localizations of β-defensin 2 expression include nasal and oral mucosa, nasolacrimal duct, ocular surface epithelium, intestinal epithelium and kidneys (Lehmann et al. 2002, Brogden et al. 2003) in response to infection and inflammation, but in our reaserch this peptide was predominantly expressed in hepatocytes. Our finding of a positive relation between inflammation cells and intensity of defensin expression in mink liver proves the stimulating role of defensin on cell proliferation as a compensatory mechanism, that agree with finding of other researchers (Murphy et al. 1993).

Discharging of HGF was basically observed in hepatocytes disposed or located focally around bile ducts and portal ducts. Besides, a dispersion of the growth factor around bile ducts and portal ducts coincides with areas where inflammation cells were found more often. This observation proves that HGF acts as a factor improving liver regeneration during damage of the organ which is confirmed by other research (Ishikawa et al. 2001, Watanabe et al. 2003, Makino et al. 2006). This can be explained by the HGF ability to stimulate the proliferation of not only parenchymatous liver cells but also of biliary 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 (Lindroos et al. 1991, Maher 1993). Among non-parenchymal cells we suggest HGF expression in endotheliocytes of veins, which, possibly proves compensatory activity of HGF during inflammation. Thereby, HGF operates as a factor promoting liver regeneration during damage (Ishikawa et al. 2001, Watanabe et al. 2003, Makino et al. 2006).

Apoptosis is genetically determined, internal cell destruction, influenced by different conditions. A reason for destruction of hepatocytes can be the activity of microorganisms (Klatskin and Conn 1993). Apoptosis of hepatocytes in all parts of parenchyma was observed, although physiologically the apoptotic process is basically going on in vein area, which means that hepatocytes are developing in periportal section of liver and slowly migrate to centrolobular area where they are degenerated (Klatskin and Conn 1993, Green 1998). It certifies about a systemic infection, because such infection is connected with infiltration of Kupffer cells and different number of neutrophils (Klatskin and Conn 1993).

From observed coherence that either at a very extensive or an average apoptosis level, there is no big difference in expression of inflammation cells whereas at a small apoptosis level – their number is a considerably lower, we can conclude that apoptotic process can also go on without the development of inflammation reaction which coincides with the data of other researchers (Green 1998, Green and Ree 1998). The relatively small apoptotic cell count obtained in liver coincides with the data shown by other scientists that apoptosis can be observed in liver during immune regulation and infection. As the obtained results did not show any correlation of apoptosis of hepatocytes with infiltration of inflammation cells, we consider that there is some other reason causing apoptosis. Klatskin and Conn (1993) and Schulte-Hermann et al. (1997) showed that such a process is characteristic of viral infections as the result of a combined effect of caspases activity and damage of cell mitochondrion that activates endonucleases and internucleosomal fragmentation of DNS is triggered. This small number of apoptotic hepatocytes can possibly be explained by the presence of Aleutian disease virus persisting in mink organism as the impact of this virus can hinder the apoptotic process by inhibiting nuclear protein p53 and caspases, which damage cell DNA.

In general, the research showed that in spite of infiltration of inflammation cells found in liver, expression of degeneration markers as matrix metalloproteinases, damage of the basement membrane, apoptosis of hepatocytes liver of minks affected by Aleutian disease virus have still maintained a considerable regeneration ability proved by an intense release of growth factors: from hepatic stellate cells (nerve growth factor receptors – NGFR p75), from cells of liver parenchyma and non-parenchyma (hepatocyte growth factors – HGF) and from periportal hepatocytes and endothelial cells (vascular endothelial growth factor – VEGF).

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