Experimental Model of Hemorrhagic Stroke: Rabbit Immunization with HL-60 Promyelocytic Cell Differentiation Factor

M. V. Gapon, S. M. Dranitsyna, N. I. Minkevich, M. A. Gruden'*, I. I. Babichenko, and I. A. Kostanyan**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 141, No. 2, pp. 237-240, February, 2006
Original article submitted October 7, 2005

Rabbits immunized with synthetic HLDF differentiation factor developed hemorrhagic stroke with thrombosis of small cerebral vessels and destruction of vascular endotheliocytes. The severity of stroke correlated with serum level of antibodies to differentiation factor. The role of different sites of HLDF molecule in the induction of clinical signs of hemorrhagic stroke was studied.

Key Words: *HLDF; immunization; experimental model of hemorrhagic stroke*

Molecular mechanisms of acute circulatory disorders developing in cerebrovascular diseases are now actively studied. However, there is still no adequate model of acute disorders in cerebral circulation: all models are based on occlusion of the carotid arteries, *i.e.* mechanical injury to large vessels [10]. An alternative approach to creation of adequate experimental models of cerebrovascular disorders in animals is injection of vasoactive protein factors. Variations in the concentrations of these factors are a peculiar marker of these conditions [12].

One of these proteins is human leukemia differentiation factor (HLDF) [3]. HLDF protein and the corresponding autoantibodies are involved in the mechanisms of development of acute disorders in cerebral circulation of hypertensive origin [6].

HLDF is a glycosylated protein with a molecular weight of 8.2 kDa [3]. It induces differentiation of actively proliferating HL-60 cells and participates in apoptosis processes. Two active fragments of HLDF are identified: six-member peptide fragment (HLDF-6) completely retaining differentiating activity of the initial protein and possessing antiapoptotic and cell-protective properties and eight-member peptide (HLDF-8) capable of inducing programmed cell death [1,2]. HLDF is present in mammalian and human blood and nervous system, while the peptide fragments of this protein are essential for cognitive functions [14]. Hemodynamic activities of differentiation factor peptides HLDF-8 and HLDF-24 modifying arterial pressure and heart rate were demonstrated [4].

We studied neuroimmunological, structural, and morphological changes in animals after active immunization with HLDF and its peptide fragments.

MATERIALS AND METHODS

The study was carried out on Chinchilla rabbits. Males (2 kg) were immunized with synthetic HLDF and its bioactive fragments HLDF-6, HLDF-8, and HLDF-24 by the standard protocol for obtaining antisera [9]. Since peptide fragments HLDF-6 and HLDF-8 are not immunogenic (because of their small size), they were conjugated with KLH (key-

Laboratory of Hormonal Regulation Proteins, M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Organic Biochemistry, Russian Academy of Sciences; *Laboratory of Neurochemistry, P. K. Anokhin Institute of Normal Physiology, Russian Academy of Medical Sciences; **Department of Pathological Anatomy, Medical Faculty of Russian University of Peoples' Friendship. ***Address for correspondence:*** iakost@mail.ibch.ru. I. A. Kostanyan

0007-4888/06/1412-0272

hole-limper hemocyanin) carrier protein with glutaraldehyde. Peptide conjugate was obtained by the standard method [9]. The blood was collected 1 month after immunization and then every 2 weeks. Serum antibodies were evaluated by ELISA every 2 weeks [9]. After manifestation of signs of neurodegenerative disorders (as a rule on day 65 of immunization) the rabbits were sacrificed under narcosis and the brain was fixed in formalin. Paraffin sections of the brain were stained with hematoxylin and eosin by the standard method and examined under a microscope.

RESULTS

Immunization of 5 rabbits with synthetic full-length HLDF resulted in the formation of polyclonal antibodies to this protein. When titers of serum antibodies reached 1:1000, the animals exhibited symptoms resembling the clinical picture of acute stroke in humans (difficult breathing, paralysis of lower limbs, paresis of cervical muscle, frequent urination). Impairment of the blood-brain barrier in these rabbits was seen from 3-fold increase in serum content of S100b neurospecific protein in comparison with intact animals. Similar changes were observed in patients with acute stroke [15].

Histological study of the brain tissues revealed disorders in cerebral circulation by the hemorrhagic type. Analysis of sections stained with hematoxylin and eosin showed pronounced perineuronal and perivascular edema in the white and gray matter. Hemorrhagic infiltration around small vessels in the subcortical nuclei indicated high permeability of these vessels (Fig. 1.). Homogeneous substance (hyalin clots) was detected in capillaries. Formation of apoptotic bodies was seen in vascular endotheliocytes (Fig. 2). The cells were oval or round, pyknotic nuclei were surrounded with a light cytoplasm rim. Capillary thrombosis caused blood stasis and led to erythrocyte hemolysis in cerebral vessels and to the development of the sludge syndrome. These changes were also detected in the cerebellum of immunized animals. Thrombosed vessels were seen at the interface of the white and gray matter of the cerebellum. According to histological analysis of cerebral tissues, small focal disorders of cerebral circulation were numerous and resulted from repeated microstrokes.

Similar morphological picture was observed in animals (n=5) immunized with HLDF-24 peptide fragment, characterized by higher immunogenic activity in comparison with full-length factor. Antibody titer reached 1:16,000. HLDF-6 and HLDF-8 peptides were not immunogenic, but immunization with their conjugates with snail hemocyanin induced production of antibodies. Clinical picture after immunization with HLDF-8-KLH conjugate was virtually the same as in animals immunized with full-length factor (hind limb paralysis, cervical muscle paresis). Autopsy showed numerous hemorrhages in the brain.

HLDF-6-KLH conjugate was very weakly immunogenic, antibody titer did not surpass 1:400, neurological symptoms were minor (slight paresis of cervical muscles), and histological analysis of brain sections after immunization indicated slight perineuronal edema without impairment of the blood-brain barrier.

Immunization with peptide homologous to HLDF N-terminal fragment and human ribosomal S21 protein (having common amino acid sequence with HLDF precursor protein [5]) induced production of antibodies possessing no cytotoxic effect. These data indirectly indicate that proteins capable of reacting with antibodies to HLDF are present in rabbit cerebrovascular endothelium; these proteins seem to be involved in the formation of the blood-brain barrier. They have an amino acid sequence

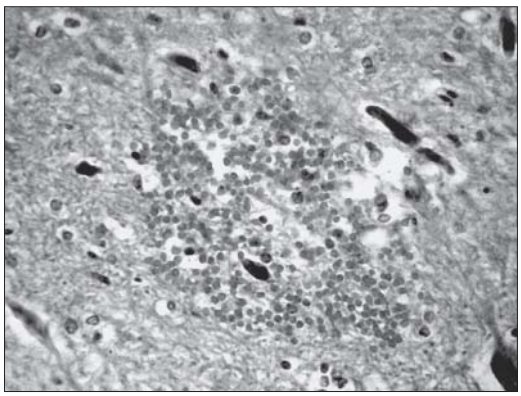

Fig. 1. Hemorrhagic infiltration in subcortical structures of the brain in a rabbit 90 days after immunization with full-length HLDF (×200).

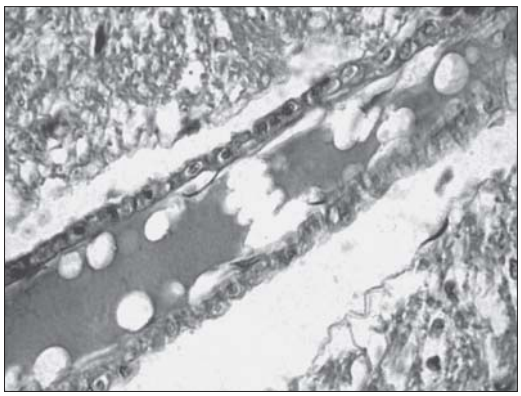

Fig. 2. Hyalin clots, apoptotic changes in endotheliocytes, and perivascular edema in cerebral vessel of a rabbit 90 days after immunization with full-length HLDF (×1000).

homologous to that of HLDF-8 (RRWHRLKE). Antibodies to C-terminal part of HLDF molecule only augment the signs of stroke. Some authors attribute cerebral ischemia and its extreme manifestation (hemorrhagic stroke) to cerebrovascular vasculites [7] (a group of diseases associated with inflammation of blood vessel walls). Early diagnosis of vasculitis in CNS is an intricate problem, because this condition has no specific symptoms and the tissues are unavailable for pathohistological studies.

Antibodies specifically staining the neutrophil cytoplasm are present in the blood of patients with Wegener's granulomatosis (the most incident form of vasculitis). The presence of these antibodies is a marker of vasculitis of minor vessels, called ANCA-associated vasculites (ANCA: antibodies to neutrophil cytoplasmic proteins). In these vasculites antibodies to neutrophil proteins disorder normal interactions between neutrophils and endotheliocytes and hence, promote vascular injuries. Three groups of proteins, to which antibodies are produced in ANCA-associated vasculites, are distinguished on the base of immunofluorescent analysis of neutrophils: cytoplasmic (C-ANCA), perinuclear (P-ANCA), and atypical (A-ANCA) proteins. C-ANCA proteins react as a rule with proteinase 3 (PR3; serine proteinase with a molecular weight 29 kDa) present in azurophilic granules of the neutrophil. P-ANCA antibodies react with myeloperoxidase constituting 5% of total protein in neutrophils. Various proteins (lactoferrin, elastase, or bactericidal protein inhibiting cell permeability) can serve as antigens for A-ANCA [13]. HLDF appears in promyelocytic leukemia cells differentiated by the neutrophilic pathway under the effect of retinoic acid, which suggests that polyclonal autoantibodies to this protein are atypical ANCA associated with impaired maturation of the neutrophil. These data indicate that antibodies to this differentiation factor are involved in the mechanisms of development of acute cerebral circulation disorders. Presumably, production of antibodies to HLDF is the cause of stroke during retinoic acid therapy of acute promyelocytic leukemia [8,11].

Animals immunized with synthetic differentiation factor develop hemorrhagic stroke with thrombosis of cerebral capillaries and destruction of endotheliocytes. The severity of clinical status correlates with blood level of antibodies to differentiation factor.

Hence, we propose an experimental model of hemorrhagic stroke, which can be used in experimental studies and clinical practice for the development of methods for the treatment of this disease. The results open new prospects for early diagnosis of pre-stroke conditions in risk-group patients.

REFERENCES

1. S. M. Dranitsyna, I. A. Kostanyan, S. G. Andreeva, *et al.*, *Bioorgan. Khim.*, **26**, No. 5, 340-351 (2000).
2. I. A. Kostanyan, M. V. Astapova, E. V. Navolotskaya, *et al.*, *Ibid.*, **26**, No. 7, 505-511 (2000).
3. I. A. Kostanyan, M. V. Astapova, E. V. Starovoitova, *et al.*, *Ibid.*, **21**, No. 4, 243-248 (1995).
4. A. N. Murashev, *Proceedings of Inter-Departmental Scientific Council on Experimental and Applied Physiology* [in Russian], Vol. 10, Moscow (2001), pp. 330-331.
5. E. V. Smirnova, A. V. Garkovenko, T. V. Rakitina, *et al.*, *Bioorgan. Khim.*, **30**, No. 2, 130-140 (2004).
6. V. V. Sherstnev, V. I. Skvortsova, M. A. Gruden', *et al.*, *Zh. Nevrol. Psikhiatr.*, Supplement: *Stroke*, No. 12, 53-59 (2004).
7. C. Fieschi, M. Rasura, A. Anzini, and M. Beccia, *J. Neurol. Sci.*, **153**, 159-171 (1998).
8. H. Fujiwara, Y. Umeda, and S. Yonekura, *Leukemia*, **9**, No. 9, 1602-1603 (1995).
9. E. Harlow and D. Lane**,** *Antibodies: A Laboratory Manual*, New York (1988).
10. D. E. McBean and P. A. Kelly, *Gen. Pharmac.*, **30**, No. 4, 431-434 (1998).
11. T. Miyoshi, T. Otsuki, K. Omine, *et al.*, *Rinsho Ketsueki*, **43**, No. 10, 954-969 (2002).
12. A. Ottani, S. Saltini, M. Bartiromo, *et al.*, *Brain Res.*, **986**, Nos. 1-2, 181-190 (2003).
13. P. Seo and J. H. Stone, *Am. J. Med.*, **11**, 739-750 (2004).
14. R. D. Sewell, M. A. Gruden, D. M. Pache, *et al.*, *J. Psychopharmacol.*, **19**, No. 6, 602-608 (2005).
15. C. G. Zimmerman-Ivol, P. R. Burkhard, J. Le Floch-Rohr, *et al.*, *Mol. Cell. Proteomics.*, **3**, No. 1, 66-72 (2004).