32 Application of "In Vivo Cryotechnique" to Immunohistochemical Analyses for Effects of Anoxia on Serum Immunoglobulin and Albumin Leakage Through Blood–Brain Barrier in Mouse Cerebellum

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Abstract

In this chapter, we present the application of "in vivo cryotechnique" (IVCT) to examination of time-dependent topographical changes of leaking proteins from blood vessels in the mouse cerebellum, to assess the blood–brain barrier (BBB). The distribution of leaking serum proteins was compared by various cryotechniques. The cryofixed cerebellar tissues were processed for the freeze-substitution method and paraffin embedding. Serial deparaffinized sections were immunostained by anti-mouse immunoglobulin G (IgG) or albumin antibody. By combination of IVCT, serum IgG and albumin were clearly localized inside of cerebellar blood vessels. In anoxic cerebellar tissues, which were partially removed from brains in the mouse skull and quickly frozen in the isopentane–propane within a minute, serum IgG and albumin were diffusely immunostained around the blood capillaries, showing leakage of the serum components through the BBB changes. Thus, IVCT revealed in vivo localization of serum components in mouse brains.

Keywords

Cerebellum • Blood–brain barrier • Serum • In vivo cryotechnique

32.1 Merit of In Vivo Cryotechnique (IVCT) to Evaluate Blood–Brain Barrier (BBB)

In the central nervous system, there is a highly selective barrier that separates the circulating blood from extracellular fluid, termed BBB. Although the functional integrity of the BBB was first measured using the in vivo injection of dyestuff [1], these dyes were found to bind with plasma proteins and lose their color intensity due to chemical reactions in the biological tissues. These technical difficulties led to the development of alternative tracer methods such as the in vivo injection of horseradish peroxidase (HRP) [2, 3], radiolabeled tracers [4], or fluorescent dyes [5]. On the other hand, immunohistochemical technique has been widely used to reveal albumin localization in extravascular matrices of rat brains [6, 7]. Transcardial perfusion of trypan blue

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was also reported to be a marker for checking alteration of the BBB integrity [8].

However, conventional fixatives could easily release loosely bound or soluble protein components and then link them to other structure elements, with which they were not originally associated [9]. A technical solution for these problems has been known to be offered with another physical cryofixation, such as quick-freezing, which would have an advantage that the physical cryofixation for animal cells and tissues can be completed within a time of milliseconds [10]. For the purpose of applying the quick-freezing method for functioning cells and tissues in vivo, IVCT was developed [11], which was to arrest the transient behavior at the highest time resolution in animal living cells and tissues by the combination of a cryoknife cooled down in liquid nitrogen and another isopentane–propane cryogen (−193 °C) (Fig. 32.1a). IVCT and following freeze-substitution fixation (FS) would be the most reliable technique to keep soluble serum proteins in the blood vessels of animal brains. In addition, the protocol is useful to obtain good immunoreactivity because antibodies easily penetrate into tissue samples through tiny ice crystals (Fig. 32.1b). In this chapter, we demonstrate immunolocalization of serum proteins in mouse cerebellum with IVCT. Precise methods and results have been reported in the previous paper [12].

32.2 IVCT for Living Animal Cerebellum

Adult mice were anesthetized with sodium pentobarbital, and their cerebellum was surgically exposed. As depicted in Fig. 32.1a, while the heart was normally beating, the cerebellum was vertically cut with the precooled cryoknife as fast as possible and simultaneously poured with the isopentane–propane cryogen [11]. The frozen cerebellar tissues were carefully trimmed out with a dental drill in liquid nitrogen and then processed for the following FS step.

32.3 Detection of Serum IgG in Blood Capillaries In Vivo

Under normal condition, IgG was immunolocalized in blood vessels of cerebellum with IVCT followed by FS (Fig. 32.1c). Judging from sizes of tiny ice crystals at low resolution by light microscopy, artificial destruction of blood vessel structures was not well recognized in molecular layers at cerebellar surface layer tissues (ML in Fig. 32.1c) and granular layers at deeper regions (GL in Fig. 32.1c). The well-preserved tissues were nearly within 200–300 μm from the cerebellar surface area, which the isopentane–propane cryogen was directly poured over and the cryoknife was closely attached to. There was no immunoreactivity for IgG outside the blood vessels, suggesting no leakage or prominent movement of serum IgG proteins during the FS method as well as the immunohistochemical staining process.

After the perfusion fixation with 2 % paraformaldehyde in phosphate buffer, there was no immunoreactivity with anti-IgG antibody in the blood vessels, due to washing out of blood during the perfusion-fixation. Thus, soluble serum proteins, such as IgG, can be preserved by the combination of our IVCT and FS method.

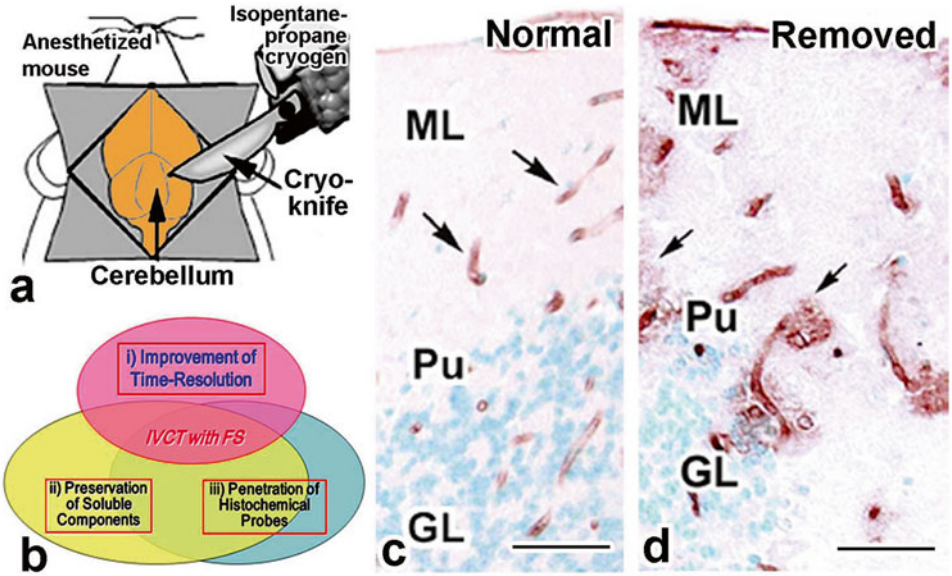

Fig. 32.1 (**a**) A schematic representation of "in vivo cryotechnique" (IVCT) for the mouse cerebellum after opening the scull; cryoknife is push into the cerebellum and simultaneously isopentane–propane cryogen (−193 °C) is poured over the cerebellum. (**b**) The schema illustrates three merits of IVCT with freeze-substitution fixation (FS). (**c**, **d**) Immunostaining of IgG in normal mouse cerebellum with IVCT (Normal; **c**) and removed cerebellum to induce hypoxic condition with quick-freezing method (Removed; **d**). *ML* molecular layer, *Pu* Purkinje cell layer, *GL* granular layer. Precise data have been reported in the previous paper (Zea-Aragón et al. [12]). Bars, **c**, **d**, 50 μm

32.4 Leakage of IgG from Blood Vessels in Anoxic Cerebellum

In the cerebellar tissues immediately after a sudden stop of cerebellar blood supply, IgG was diffusely immunostained around blood vessels (Fig. 32.1d). In such extravascular regions, there was no blood cell, indicating that no bleeding nor inflammatory reactions had happened around there. The distribution pattern of the IgG-immunostaining products was sometimes fan-shaped, indicating the leakage of serum IgG from some restricted points of the blood vessels. These findings suggest that the quick-freezing and freeze-substitution method applied in this section for immunohistochemistry enabled us to detect the functional damage of the BBB.

The immunostaining pattern of albumin was also observed to be similar to that of IgG, being a fan-shaped leakage around blood capillaries. The albumin immunoreaction products were observed to be exclusively localized within the blood vessels in cerebellar tissues under normal blood flow conditions, as prepared by IVCT.

32.5 Concluding Remarks

The combined procedure, the IVCT and FS method, presented here would be useful for clarifying time-dependent and native distribution of leaking serum proteins through damaged BBB under various pathological conditions.

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