Improved cryosections and specific immunohistochemical methods for detecting hypoxia in mouse and rat cochleae

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Summary
The present study was undertaken to develop an improved cryoembedding method for analysis of mice and rat cochleae, which permits high-quality cryosections and preserves overall structure and cellular resolution as shown by hematoxylin/eosin staining. The preservation of morphology and antigenicity is mandatory to achieve optimal results. A total of 20 male cd/1 mice and 14 male Sprague-Dawley rats were used in experiments for optimization of preservation, fixative, decalcification, embedding and cryosectioning of cochleae from adult and aged rodents. In addition, a novel immunohistochemical procedure (using HydroxyprobeTM-1 kit) was developed for detecting regions of hypoxia in mice and rat cochlea. This method employs a primary fluorescent-conjugated monoclonal antibody directed against pimonidazole protein adducts that are created in hypoxic tissues. Subsequent studies of hypoxia inducible factor-1α (HIF-1α) by immunofluorescence in the cochlea of these animals were performed in order to confirm that immunochemical detection of pimonidazole protein is representative of a hypoxic environment. We conclude that the present method results in high-quality cryosections of cochlear tissues presenting good anatomical and histological preservation. Furthermore, our optimized procedures provide novel tools for the investigation of neuro-sensory-epithelium in physio-pathological situations associated with hypoxia and/or ischemia, such as inner ear development, plasticity, regeneration and senescence.

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Introduction

The cochlea is a small but complex anatomical and functional structure consisting of the stria vascularis (SV), the spiral ligament (SLg) and the organ of Corti (OC). The inner ear has some peculiar properties owing to the complexity of interactions between neural cells and sensory epithelium forming the sensory organ of hearing. Furthermore, changes along the tonotopic axis of the cochlea have been characterized and associated with electrophysiological properties of neurosensory epithelium (Spicer and Schulte, 1996; Judice et al., 2002).

Hypoxia/ischemia is an important pathogenic factor for inner ear disease. It has been reported that perinatal and postnatal hypoxia and asphyxia may produce cochleosaccular abnormalities in the human inner ear resulting in severe bilateral sensorineural hearing loss (Attias et al., 1990; Orita et al., 2002). An important transcription factor involved in the signaling and adaptation of hypoxia/ischemia is the hypoxia inducible factor (HIF-1) (Semenza, 2000). The biological activity of HIF-1 is determined by the expression and activity of the HIF-1α subunit. The regulation of HIF-1α activity in the different anatomical structures of the inner ear and its effect on genes involved in oxygen homeostasis regulation (Gross et al., 2003), including energy supply, proliferation and cell death, are therefore of considerable interest.

Cryosections and immunochemical analysis of inner ear tissues have become commonly used techniques for exploration of cochlear structure (Spicer et al., 1999; Spicer and Schulte, 2005; Teufert and Linthicum, 2005), in development (Morsli et al., 1998), degeneration after treatment (Hong et al., 2006; Kanzaki et al., 2006) and regeneration (Lee and Warchol, 2005; Yamasoba and Kondo, 2006), noise exposure (Chen and Fechter, 2003; Pourbakht and Yamasoba, 2003) or ageing (Löwenheim et al., 1999; Jiang et al., 2006). However, owing to the structural heterogeneity of the cochlea, achievement of high-quality cochlear sections remains a technical challenge.

Several studies have shown a sustained interest in development of techniques for detecting hypoxia. An in situ technique was developed by Chapman et al. (1981), which first proposed 2-nitroimidazole binding as a marker for tissue hypoxia. This technique, which allowed a simple detection of hypoxic cells in vitro and in vivo, has been widely used in tumor tissue, and works well for the detection of hypoxia in various tissues under normal and pathological conditions.

In our laboratory, we have developed an improved embedding method in order to achieve high-quality cryostat sections of cochleae with improvements for detailed cellular identification and anatomical detail detection, coupled with hypoxia immunochemical analysis. Here, we describe how to obtain high-quality sections, visualize them using a rapid hematoxylin/eosin staining technique, and then use them for further analysis with antibodies and confocal microscopy. This offers an easy and complete visualization of all the cochlear structures by differentially labeling nuclei and cytoplasm of cells from different origins, such as epithelial cells, neural cells and fibrocytes. Sometimes a more precise and specific qualitative and/or quantitative analysis of the sensory epithelium is desirable in order to understand the specific effects of hypoxia (Samoszuk et al., 2004). Raleigh et al. (1987) demonstrated that hypoxia could be detected in several animal tissues by immunohistochemistry using antibodies that recognize hypoxia marker adducts produced by exogenous administration of pimonidazole. Therefore, we have developed the use of this technique in cochleae of mice and rats. Subsequent studies of HIF-1α immunolocalization in the cochlea of these animals have been achieved in order to confirm that immunochemical detection of pimonidazole protein can be representative of a hypoxic environment.

Materials and methods

Animals

Experiments were performed on male Sprague-Dawley (SD) rats (n = 14) (Centre d’élévation Dépré, St-Doulchard, France) and cd/1 outbred mice (n = 20) (Charles River Laboratories, Fr) of different ages, weighing from 220 to 240 and 20 to 40g, respectively. Animals were housed in living quarters with controlled temperature (21 ± 1°C) and light (12-h light/12-h dark cycle, with the light switched on at 7 a.m.), with free access to food and water. All experimental procedures were carried out in strict accordance with European Community guidelines (86/609/EEC) for the care and use of laboratory animals.

Detection of tissue hypoxia by pimonidazole adducts

Pimonidazole (1-((2-hydroxy-3-piperidinyl) propyl)-2-nitroimidazole hydrochloride), administered
in vivo, reliably binds to tissue at oxygen tensions below 10 mmHg (Kennedy et al., 1997), where it can be detected by immunohistochemistry using a specific anti-pimonidazole antibody. Pimonidazole (Hydroxyprobe™-1, Chemicon International, Inc., Temecula, CA) was administered intravenously via one of the tail veins at a dose of 60 mg/kg of body weight, in phosphate-buffered saline (PBS, pH 7.4) 60 min prior to killing the mice or rats and processing the cochleae as described below. Cochlea sections from animals not pre-treated with pimonidazole were used as a negative control. A second type of negative control for pimonidazole labeling was the use of another mouse strain (C57/BL6), which we have used previously as a model of hearing loss (Riva et al., 2005).

**Cochlea preparation**

All animals were sacrificed by decapitation and both cochleae from each animal were dissected and perfused with a fixative solution containing 4% paraformaldehyde in phosphate-buffered saline (pH 7.2–7.4) for 3 h, at room temperature. The apical portion of the bony cochlea was gently opened using one point of a Dumont forceps, to allow the fixative to perfuse through the tissues. After rinsing (2 × 30 min in PBS), the cochleae from mice were decalcified overnight and from rats for two days in EDTA solution (Microdec®; Microm, France), then soaked in 20% sucrose solution for 8 h and finally overnight in 10% sucrose. The cochleae were placed in Tissue-Tek OCT compound (Tissue-Tek®, Labonord, France) for cryostat sections. The cochleae were oriented to obtain cross-sections of the organ of Corti. Five micrometer-thick sections were cut by cryostat, mounted on SuperFrost® Plus slides (Menzel-Glaser, Braunschweig, Germany) coated with gelatin (0.5% gelatin and 0.05% chromium potassium sulfate) and then post-fixed in 4% paraformaldehyde for 10 min. A maximum of ten slides per cochlea were produced, with several sections on each slide, so that each slide contained representative sections of the entire cochlea.

**Hematoxylin/eosin staining**

In order to demonstrate the tissue integrity after this cryosectioning protocol, we used hematoxylin/eosin staining. Depending on the desired intensity of the final staining, incubation times with hematoxylin can be varied. The protocol we used was: incubation with hematoxylin solution (Ral France), 10 min (giving a lavender color), rinsing in water 2 min, then incubation in 2% eosin solution (Sigma® France) for 2 min. After a brief water wash, sections were dehydrated through an ascending alcohol bath series (50%, 75%, 95%, 100%, 2 min in each), cleared in xylene (Labonord, France) for 2 min, then slides were allowed to air dry and mounted in Eukitt (Labonord, France).

**Immunocytochemistry**

Cryostat sections, prepared as described previously, were incubated for 30 min at room temperature with PBS containing 5% bovine serum albumin (PBS-BSA) to prevent non-specific labeling. The specimens were then incubated for 16 h at 4 °C with primary antibodies. These were: hydroxyprobe™-1 mouse monoclonal antibody IgG1 (hydroxyprobe-1 Mab-1, Chemicon Int, CA) (Kennedy et al., 1997) diluted 1:200, and rabbit polyclonal anti-HIF-1α (H206, Santa Cruz Biotechnology, CA) diluted 1:1000. Slides were washed three times in PBS-BSA and exposed to secondary antibodies for 4 h at room temperature. These were fluorescein isothiocyanate (FITC)-conjugated donkey F(ab’)2 anti-mouse antibody or anti-rabbit antibody (both from Jackson Immunoresearch, West Grove, PA), both diluted 1:400. Nuclei were counterstained with DAPI (Calbiochem®). Slides were then air-dried, mounted in Mowiol (Calbiochem®) and stored at 4 °C. All dilutions and thorough washes between steps were performed using PBS-BSA unless otherwise stated. Replacing the primary antibody with IgG1 or rabbit immunoglobulin serum fraction acted as a control for the specificity of the immunological reaction.

**Microscopy and analysis of cochlea sections**

Slides were examined using a Leica DMR – microscope (Leica Microsystems AG, Wetzlar, Germany) equipped for epifluorescence with 40 × and 100 × oil-immersion objectives. Images were acquired using a cooled CDD Photometrics Coolsnap camera (Roper Scientific, Tucson, AZ) and the image files were processed using Photoshop software (Adobe, San Jose, CA). The relationship between the degree of hypoxia (indicated by pimonidazole staining) and HIF-1α protein immunolocalization was assessed semi-quantitatively, as follows. Pimonidazole labeling intensity was classified as absent, moderate or strong. Parallel sections had been stained with hematoxylin/eosin and labeled for HIF-1α and each cochlear region was analyzed. HIF-1α immunolabeling was classified according to signal abundance: absent, moderate or strong. The average evaluation of two fields
seen, using a 10 × objective lens, from each animal was evaluated.

Statistics

The number of histological observations is equal to the number of cochleae tested, i.e. cd/1 mice \( n = 40 \), Sprague-Dawley rats \( n = 28 \). The non-paired Student \( t \)-test was used to compare collected data regarding co-labeling with pimonidazole adducts and for HIF-1α. The difference was considered significant at \( p < 0.05 \).

Results

Figure 1 illustrates a representative section of a young (4 week old) cd/1 mouse cochlea, which was used as a reference anatomy slide for the analysis of murine cochlea. This section clearly shows an intact organ of Corti resting on the basilar membrane (BM), well demarcated between the scala media and scala tympani. The spiral ligament (SLg), stria vascularis (SV), spiral limbus (SL), spiral ganglion (SG), Reissner’s membrane (RM) and tectorial membrane (TM) are also clearly defined. The typical organ of Corti structure is composed of inner hair cells (ihc), outer hair cells (ohc) and supporting cells (sc). Cells of Reissner’s and tectorial membranes were lightly stained with hematoxylin/eosin, while stria vascularis (SV) and neuron cells from Rosenthal’s canal (R) and the spiral ganglion were more darkly stained. The hair cells and their nuclei were clearly detected. The organ of Corti is linked on one side to the spiral limbus by the tectorial membrane and to Rosenthal’s canal and the spiral ganglion. On the other side, it is linked to the lateral wall composed of the stria vascularis (SV), the spiral prominence (SP) and the spiral ligament (SLg) containing types I (a), II (b), III (c) and IV (d) fibrocytes. Fibrocytes of the spiral ligament are divided into four cell types based on structural features and general localization according to Spicer and Schulte (1996). The detailed organization of the spiral ligament is summarized in the diagram shown in Fig. 2. The cryosection technique described here thus facilitated preservation of intact cochlear histology as seen by hematoxylin/eosin staining.

To detect the presence of hypoxia we assessed the detection of pimonidazole adducts in the cochlea injected with the bioreductive dye and also mapped HIF-1α protein immunolocalisation. All negative control preparations for pimonidazole adducts were completely unlabeled (data not

Figure 1. (A) Light micrograph of a cross-section of a hematoxylin/eosin stained cd/1 mouse cochlea, showing the organ of Corti and the lateral wall structures. The organ of Corti typical architecture is composed of the inner hair cells (ihc), the outer hair cells (ohc) and the supporting cells (sc), all resting on the basilar membrane (BM). The lateral wall is composed of the stria vascularis (SV), the spiral prominence (SP) and the spiral ligament (SLg) containing type I (a), type II (b), type III (c) and type IV (d) fibrocytes. Scale bar = 50 μm. (B) Light micrograph of a cross-section of a hematoxylin/eosin stained cd/1 mouse cochlea, showing the organ of Corti composed of inner hair cells (ihc), outer hair cells (ohc), supporting cells (sc) and the nervous system. Schwann cells and myelinated nerve fibers that fill Rosenthal’s canal (R) make the junction between hair cells and spiral ganglion (SG). SL, TM, respectively denote spiral limbus and tectorial membrane. Scale bar = 50 μm.
shown). Table 1 summarizes cochlea characteristics after Hydroxyprobe™-1 and HIF-1α immunolabeling. In aged (12 weeks old) cd/1 mice cochleae, hypoxic regions were demonstrated by an extensive labeling in the cytoplasm of the cells (Fig. 3A and B) in the modiolus (M), Rosenthal’s canal (R), spiral ganglion (SG) and the stria vascularis (SV). In addition, the HIF-1α protein was found to be slightly more intensely labeled in the cochlea of cd/1 mice aged 12 weeks in comparison to cd/1 mice aged 4 weeks; however, the difference was not statistically significant. The HIF-1α labeling confirmed the presence of the hypoxia-specific marker assessed by Hydroxyprobe™-1 adducts detection, as shown in the spiral ganglion. Furthermore, while negative or weak immunolabeling of HIF-1α was found in young adult Sprague-Dawley rats, HIF-1α was intensely labeled in 18-months-old rats (difference statistically significant, p<0.05), but with a different pattern of labeling and subtle differences in its regional distribution (Table 1). Different intensities of HIF-1α protein immunolabeling were seen at different levels in the spiral ganglion of aged Sprague-Dawley rats and cd/1 mice as seen in Fig. 4.

**Discussion**

The purpose of this study was to develop a standardized protocol for rat and mouse cochlea preparation for common immunohistochemical analysis and to detect regions of hypoxia in cochlear tissues. We therefore developed an improved embedding method, which provides high-quality cryosections of cochlear tissues presenting good anatomical and histological preservation as well as preservation of antigenicity that is crucial for successful immunohistochemistry. Previous studies have reported protocols for cochlear preparation, but none have shown direct application for immunohistochemistry (Jagger et al., 2000; Whitlon et al., 2001; Hurley et al., 2003).

The hematoxylin–eosin-staining protocol allowed us to clearly distinguish the anatomical details of the cochlea. These anatomical structures included the organ of Corti formed by the oHC, the iHC and the pillar cells (PC); the lateral wall formed by the stria vascularis (SV) and the spiral ligament (SLg);

**Table 1. Summary of immunohistochemical hypoxia detection**

<table>
<thead>
<tr>
<th></th>
<th>Cochlear tissues</th>
<th>cd/1 mice 4 weeks old (n = 20)</th>
<th>cd/1 mice 12 weeks old (n = 20)</th>
<th>SD rats 2 months old (n = 7)</th>
<th>SD rats 18 months old (n = 7)</th>
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<tr>
<td>Hydroxyprobe TM-1</td>
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*M: modiolus; SG: spiral ganglion; SLg: spiral ligament; SV: stria vascularis; HC: hair cells.

†score: –, negative labeling; +, 1–20% of positive cells; ++, 21–59% of positive cells; ++++, 60–80% of positive cells; ++++, >80% of positive cells; nd: not done.
the basilar membrane (BM); the tectorial membrane (TM); the spiral limbus (SLm); the spiral ganglion (SG) and the spiral lamina (SL). It provides information about the width and the constitution of the stria vascularis, the arrangement of the fibrocytes forming the spiral ligament, as well as...
the aspect of the hair cells and their supporting cells. The technique is simple and can be carried out very quickly. Nuclear heterochromatin and the cytoplasm of cells rich in ribonucleoprotein stained blue with the hematoxylin. The cytoplasm of cells with minimal amounts of ribonucleoprotein tends to be lavender in color, whereas the mature red blood cells seen in the stria vascularis, which are devoid of RNA, stain red. In our protocol, we reduced the hematoxylin staining time and conserved the eosin counterstaining in order to obtain lighter colors and therefore to optimize the visualization of fibrocytes and stria vascularis.

In addition, the hematoxylin–eosin staining protocol can be used to detect cochlear histopathologies, as previously reported in the case of age-related changes in the murine cochlear lateral wall (Ichimiya et al., 2000) and in the case of idiopathic sudden sensorineural hearing loss (Merchant et al., 2005). The fact that hypoxia and/or ischemia may play a role in pathogenesis of the inner ear disease (tinnitus or hearing loss) emphasises the importance of radical forming processes in this phenomenon (Riva et al., 2006). Therefore, this demonstrates the need to investigate the hypoxia status in the inner ear. In our study, we found a differential, region-specific pattern of hypoxia detected by the HydroxyprobeTM–1 kit and HIF-1α activation in cd/1 mice and in Sprague-Dawley rats. The most hypoxic region with the most intense HIF1α labeling was observed in cd/1 mice aged 12 weeks, and was found in the spiral ganglion, the modiolus and the stria vascularis. In Sprague-Dawley rats aged 18 months the most intense HIF1α labeling was found in the hair cells.

Since HIF-1α regulates transactivation of several genes in response to hypoxic conditions (Semenza, 2000), the presence of stabilized or over-expressed HIF-1α protein is a sign of a hypoxic environment. The presence of HIF-1α in hypoxic regions was confirmed by pimonidazole labeling, as recommended by Samoszuk et al. (2004) (Table 1). Although Janssen et al. (2002) reported that HIF-1α overlapped very little with pimonidazole binding in tumor tissue, our observations are in agreement with Lee et al. (2001), who demonstrated colocalization of pimonidazole and HIF-1α in normal embryonic tissue. In fact, the molecule of pimonidazole binds specifically and covalently to proteins contained in hypoxic viable and metabolically active cells (Raleigh et al., 1999). This reaction occurs only when the oxygen pressure in the cells is lower than 10 mmHg (Raleigh et al., 1999; Rijken et al., 2000). Therefore, we believe that detecting hypoxia in situ by pimonidazole labeling should be confirmed by HIF-1α labeling, and vice versa. If both results are consistent, this becomes a cogent demonstration of the presence of a hypoxic environment in a specific tissue. In addition, these findings illustrate that the HIF-1α system is operating in the cochlea and raises the possibility that HIF-1α expression allows identification of hypoxic cells and assessment of the relationship between a hypoxic response and cellular morphology. Using these two methods for detection of hypoxia (hydroxyprobeTM–1 and HIF-1α labeling), we conclude that these procedures provide improved methods for detecting hypoxia and hypoxia regulation in mouse and rat cochlea and could be valuable tools in the study of hypoxia/ischemia in rat and mouse models of disease.

Acknowledgments

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