—Short Review—

Histochemistry for Placenta Research: Theory and Application

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Abstract

Histochemical techniques have contributed significantly to advances in placental biology and cell biology. In this mini-review, we describe recent advances in histochemical technologies and show how these technologies can profoundly improve our understanding of placenta morphological function related to health and disease. Fundamental theories and applications of five separate methods discussed here are 1) tissue-based polymerase chain reaction by laser microdissection, 2) a novel antigen retrieval method using citraconic anhydride plus heating, 3) immunohistochemical detection of Lewis-related antigen expression and galectin-1 binding in the human placenta, 4) confocal microscopy analysis of IgG transport in placental trophoblasts, and 5) high-resolution immunofluorescence and correlative microscopy using ultrathin cryosections in placental research. This review article is based on a presentation given in a workshop entitled *Histochemistry: Theory and Application* at the 12th International Federation of Placenta Associations Meeting held in Kobe, Japan, on September 9, 2006.
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Introduction

Histochemistry is a powerful and diverse set of methods directed toward obtaining spatial and temporal information concerning the expression and distribution of biomolecules *in situ*. Histochemistry can provide information that cannot be gained readily with biochemical or morphological methods alone. Histochemical techniques have contributed significantly to advances in placental biology and cell biology.

This review article is based on a presentation given in a workshop entitled *Histochemistry: Theory and Application* at the 12th International Federation of Placenta Associations (IFPA) Meeting held in Kobe, Japan, on September 9, 2006. The goal of the presentation was to describe recent advances in histochemical technologies and to show how these technologies can profoundly improve our

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understanding of placenta morphological function related to health and disease. Hidetaka Eguchi presented the principles of tissue-based polymerase chain reaction (PCR) by laser microdissection for long-term preserved formalin-fixed, paraffin-embedded (FFPE) tissue specimens. Shigeki Namimatsu demonstrated a novel antigen retrieval (AR) method in which the effects of formalin fixation were reversed with citraconic anhydride plus heating. Udo Jeschke showed immunohistochemical detection of Lewis-related antigen expression and galectin-1 binding in the human placenta. Renate Fuchs gave a lecture on IgG transport in placental trophoblasts by confocal microscopy. Finally, John M. Robinson presented high-resolution immunofluorescence and correlative microscopy using ultrathin cryosections in placental research. These technologies discussed here offer valuable insights into trophoblast research.

Improvement of PCR and Reverse Transcription-PCR Amplification for Molecular Analyses with Long-Term Preserved FFPE Tissue Specimens

DNA and RNA extracted from archival tissue specimens have become invaluable sources of material for molecular biological analysis in retrospective studies, especially for rare diseases or environmental toxicology. Successful amplification with PCR/reverse transcription (RT)-PCR is essential for molecular analyses of DNA/RNA extracted from archival FFPE tissue. Various trials to overcome the problems in amplification of fragments longer than 100 bp for DNA and 70 bp for RNA have been performed, including changes of fixative, such as methanol, prolonged proteinase K treatment, and gradual dehydration. Of these trials, that by Shi and his colleagues found that heat treatment of FFPE tissue specimens at alkaline pH improved efficiencies of DNA extraction and PCR amplification, using an adaptation of the basic principles of AR[1,2].

At the 2006 IFPA Meeting Eguchi presented an improved method for efficient PCR and RT-PCR amplification using the DNA/RNA that were already extracted from archival FFPE tissue[3]. Using control DNA from formalin-fixed cancer cells as a test sample, Eguchi and colleagues searched for conditions of heat treatment of DNA with real-time PCR. Incubation in 25 mM borate buffer (pH 11.0) at 100℃ for 30 minutes followed by ethanol precipitation was effective in restoring DNA as a template. The heat treatments provided 2- to 50-fold increases in the PCR amplification efficiency of amplicons with sizes of 128, 197, and 258 bp. The treatment was then applied to DNA samples from laser-microdissected FFPE lung cancer tissue that had been stored for 4 to 18 years: the heat treatment reduced the amount of DNA required for successful PCR of amplicons ranging from 73 to 258 bp. Furthermore, various short tandem repeat sequences were effectively amplified by heat treatment of DNA samples that did not yield any amplicons when not treated. Importantly, the heat treatment was also effective for DNA samples from unbuffered FFPE tissue specimens. The heat treatment of RNA also improved the efficiency of RT-PCR[3]. Incubation in 25 mM of citrate buffer (pH 4.0) at 70℃ for 30 minutes before the RT reaction was found to be optimal. The heat-treated RNA markedly enhanced the efficiency of RT-PCR of fragments ranging from 61 to 275 bp. These improvements may be due to the elimination of chemical modification of nucleic acids generated by fixation with formalin.

The tissue-based PCR techniques presented by Eguchi are simple and cost-effective and require no special apparatus. Therefore, these techniques will be applicable for various molecular analyses of DNA/RNA samples from long-term preserved FFPE specimens.

Presentation of a Novel Method of AR Using Citraconic Anhydride for Immunohistochemical Staining

Formalin fixation masks some tissue antigens by protein cross-linking. Namimatsu presented a novel AR method in which the effects of formalin fixation were reversed with citraconic anhydride plus heating at the 2006 IFPA Meeting[4].

FFPE tissues from various organs (tonsil, ovary,

skin, lymph node, stomach, breast, colon, lung, thymus, and placenta) were sectioned, deparaffinized, placed in a kitchen electric pot containing 0.05% citraconic anhydride solution, pH 7.4, and heated at 98℃ for 45 minutes[4]. The sections were then washed in buffer solution and immunostained using a labeled streptavidin-biotin method in an automated staining system. All formalin-fixed tissues demonstrated specific staining comparable to that in fresh-frozen tissues and were markedly more enhanced than after standard AR methods. Different classes of antigens, including cellular markers, receptors, and cytoplasmic and nuclear proteins, showed enhanced reactions. Also, several difficult-to-detect antigens such as CD4, cyclin D1, granzyme B, bcl-6, and lamda chain, revealed distinct staining. The mechanism underlying the new AR method described above is believed to be the reaction of formaldehyde mainly with the lysyl residues and the formation of intramolecular cross-links[5]. The free amino groups of proteins form aminomethylol groups that then combine with other functional groups, such as phenolic, imidazole, and indole groups, to form methylene bridges. Citraconic anhydride reacts with the free amino groups of proteins and replaces the positively charged NH_3^+ groups of lysyl residues with negatively charged carboxyl groups. At pH 7.4 in hot water, adducts of citraconic anhydride and amines will undergo slow hydrolysis, which will liberate the original amines. To exclude the possibility that the new AR method causes loss of hormones and enzymes, a number of hormones and enzymes that could be easily lost (e.g., growth hormone, adrenocorticotropic hormone, human chorionic gonadotropin, prolactin, human placental lactogen, insulin, diffusible proteins [lactoferrin and S-100] and enzymes [prostatic acid phosphatase and placental alkaline phosphatase]) were also examined. None of these substances showed a loss.

The AR method developed by Namimatsu et al. provides efficient AR for successful immunostaining of a wide variety of antigens under an optimized condition. It also allows standardization of immunohistochemistry for formalin-fixed tissues in pathology laboratories, reducing inter-laboratory discrepancies in results for accurate clinical and research studies.

Immunohistochemical Detection of Oligosaccharide Expression and Galectin-1 Binding in the Human Placenta

The N- and O-linked oligosaccharide chains of glycoproteins are known to play important roles in inflammatory processes, lymphocyte homing, and initial gamete binding. Lewis antigens belong to a group of carbohydrate antigens that mediate cellular adhesion through interaction with selectins[6,7]. The Thomsen-Friedenreich (TF) glycotope (Galβ1-3GalNAcα-O-) is a carcinoma-associated antigen[8]. Galectin-1 (gal-1), a member of the mammalian β-galactoside-binding proteins, preferentially recognizes Galβ1-4GlcNAc sequences of several cell surface oligosaccharides[9].

Jeschke Immunohistochemically demonstrated the expression of Sialyl Lewis a (sLe^a), Sialyl Lewis x (sLe^x) and Lewis y (Le^y) in term placental tissue sections obtained from cases of normal, intrauterine growth retardation (IUGR), preeclamptic, and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome pregnancies at the 2006 IFPA Meeting. sLe^x was significantly increased in placentas from IUGR pregnancies and moderately increased in placentas from pregnancies with preeclampsia compared with placentas from normal pregnancies. Additionally, sLe^x was found in the syncytiotrophoblast (STB) of term placentas but without any significant differences in staining intensity between normal and pathological cases. sLe^a expression was restricted to amnion epithelia in all specimens. Finally, in cytotrophoblasts and villous endothelial cells, Le^y expression was significantly upregulated in IUGR and HELLP pregnancies.

Jeschke also showed the identification of the TF epitope and its putative carrier protein mucin 1 (MUC1) in human placenta tissue and binding of gal-1 to the same tissue[10,11]. In the first trimester of pregnancy, TF antigen and MUC1 at the apical side of the STB directed towards the maternal blood were strongly expressed[10]. This expression was consistent in the second trimester of pregnancy and,

to a lesser degree, in the third trimester. In addition, TF antigen and MUC1 on extravillous trophoblast cells in the decidua during the first and second trimesters of pregnancy showed positive staining[11]. Jeschke also histochemically revealed that the gal-1 recognized appropriate glycotopes on the STB and extravillous trophoblast layer from human placenta[9]. Gal-1 binding was diminished by the TF-disaccharide (Galβ1-3GalNAc-) conjugated to polyacrylamide[12].

These histochemical findings suggest that down-regulation of sLe^x in the extravillous trophoblast layer is associated with IUGR and preeclampsia. Furthermore, Le^y, which was recently shown to act as a potent angiogenic factor, is upregulated in placental villi in conditions associated with placental malperfusion. Gal-1 inhibited BeWo cell proliferation in a concentration-dependent manner. Similar antiproliferative effects were also observed with an anti-TF monoclonal antibody (clone A78-G/A7). Therefore, ligation of Galβ1-4GlcNAc and Galβ1-3GalNAc epitopes on trophoblast cells may have regulatory effects on cell proliferation[13].

IgG Transport in Placental Trophoblasts

For its immune defense the human neonate depends on maternal IgG antibodies that are acquired from the mother through placental transport. At term, maternal-to-fetal IgG transmission involves transfer across two cell layers, the villous STB and fetal endothelial cells. Both cells types express the human neonatal Fc-receptor hFcRn, composed of a transmembrane alpha-chain and beta2-microglobulin (b2m)[14,15]. In addition, fetal endothelial cells contain high mRNA and protein levels of another Fc-receptor, FcRIIb2[16]. The importance of hFcRn in overall IgG transfer from the maternal into the fetal circulation has been clearly demonstrated in the *ex vivo* perfused placenta[17].

To characterize the role of hFcRn in IgG transport in term villous STB and—as a model for STB—in polarized, trophoblast-derived BeWo cells, Fuchs et al. have performed transport studies and applied immunoelectron and fluorescence microcopy, respectively. Fuchs presented data on IgG transport in placental trophoblasts by confocal microscopy at the 2006 IFPA Meeting. In BeWo cells, hFcRn mediated apical IgG recycling and apical-to-basolateral IgG transcytosis[18]. Confocal microscopy revealed hFcRn/b2m staining at the apical plasma membrane, in endosomes below the apical membrane, the perinuclear area and near the basolateral membrane. Regardless of the region examined, many hFcRn/b2m-containing endosomes were also positive for the early endosomal antigen EEA1 and for the transferrin receptor. After continuous apical endocytosis, IgG was found in compartments containing hFcRn and EEA1 in all regions of the cell. Neither hFcRn/b2m nor IgG was detected in LAMP2-positive late endosomes and lysosomes. These findings are in contrast with the IgG localization in STB *in situ*. As shown previously, villous STB synthesizes b2m protein[19]. Consistent with the association of hFcRn alpha-chain with b2m found by immunoisolation[15], the alpha-chain also co-localized with b2m at the apical plasma membrane and in intracellular vesicles. Although IgG was found in endosomes containing hFcRn in the apical and basal cytoplasm, most of the IgG was detected in LAMP2-positive multivesicular bodies. Furthermore, in terminal villi, the density of hFcRn-positive endosomes was higher in fetal endothelial cells than in STB.

The continuous exposure to high IgG concentrations in the maternal blood suggest that Fc-receptors in STB are saturated and result in IgG transport to lysosomes and in IgG degradation. However, at low concentrations in BeWo cells, IgG is efficiently recycled and transcytosed via hFcRn, thus preventing its degradation. Transport across the fetal endothelium may be accomplished by hFcRn and by FcRIIb2.

High-resolution Immunofluorescence and Correlative Microscopy in Placental Research

High-resolution immunofluorescence and correlative microscopy are valuable tools that can be used to address questions related to placental biology when a high degree of spatial resolution is critical. A key aspect of the methods discussed by

Robinson is the use of ultrathin cryosections (50 to 100 nm thick) as the substratum for immunolabeling.

The use of ultrathin cryosections for immunocytochemistry at the electron microscopic level has been in evidence for several years following the pioneering work of Tokuyasu[20]. However, ultrathin cryosections have seldom been used for fluorescence microscopy. New methods have been developed for using ultrathin cryosections for immunofluorescence microscopy[21]; practical and theoretical advantages to this approach have been reported[22,23] and were discussed in this workshop. The major advantage of the approach is increased resolution in the *z*-dimension. This is especially important in studies using solid tissues, such as placenta; solid tissues must be sliced into sections to be useful for microscopy. In routine microscopy, tissue sections are generally 5 to 20 μm thick. In immunofluorescence microscopy, such sections would generally have large amounts of out-of-focus fluorescence, which would limit the information that can be derived from these "thick" sections. With ultrathin cryosections, all of the fluorescence signal must be derived from within these physical sections. In addition, there is essentially no out-of-focus fluorescence signal in these extremely thin sections. Another technology used to reduce out of focus fluorescence is optical sectioning; this is the basis of confocal microscopy. In commercially available confocal microscopes, resolution in the *z*-dimension is, at best, 500 nm and is often 1,000 nm or greater. In co-localization studies, where two or more antigens are localized using differently colored fluorochromes for detection, the possibility of false co-localization is less with ultrathin cryosection than with confocal microscopy. This is particularly true for small subcellular structures that may be stacked in the *z*-dimension. The use of ultrathin cryosections in conjunction with indirect immunofluorescence localization of several different antigens led to the recent discovery of a previously undescribed organelle in endothelial cells in the human placenta[16]. Aspects of this study were presented in this workshop.

Another important feature associated with ultrathin cryosections is that they permit correlative microscopy. Correlative microscopy is the examination of the same specimen (e.g., tissue section, cell, subcellular structure) with two or more imaging methods. For example, combining immunofluorescence and immunoelectron microscopy to examine the same subcellular structures is a form of correlative microscopy. Ultrathin cryosections (50～100 nm) are in the same thickness range as conventional plastic sections that are routinely used in electron microscopy. This type of correlative microscopy is a powerful technique that can be used when immunofluorescence detection is not sufficient to answer the question being addressed, such as when higher degrees of resolution and identification are required. For this correlative approach to work, detection systems compatible with both fluorescence and electron microscopy must be available. A reagent that is compatible with both methods is FluoroNanogold, which is a bifunctional reagent containing a fluorochrome and a gold-cluster compound. The fluorochrome can be imaged directly with the fluorescence microscope, and the gold-cluster compound can be observed with the electron microscope following enlargement with a silver enhancement reaction. Coupling ultrathin cryosections with the FluoroNanogold detection system high-resolution correlative microscopy has been performed with placental tissue. These methods have been reported in great detail[24] and aspects of the method were presented in this workshop.

High-resolution immunofluorescence and correlative fluorescence and electron microscopy using ultrathin cryosections are powerful techniques. These methods have been used successfully in studies of the placenta. Future use of these methods is expected to yield new insights into placental biology.

Future Directions

The methodological advances and the increased understanding of placental biology discussed in the workshop entitled *Histochemistry: Theory and Application* at the 2006 IFPA meeting in Kobe, Japan,

highlight the continued importance of histochemistry. As noted earlier, histochemistry and its subspecialties, such as immunohistochemistry, enzyme histochemistry, and *in situ* hybridization, can provide information not readily obtained when biochemical, immunochemical, or morphological methods are used alone. The advances in methodology presented in this workshop will likely be very valuable in enabling new studies to be conducted in the placenta and in reproductive biology in general. These anticipated studies will provide greater insight into the placenta in health and disease.

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