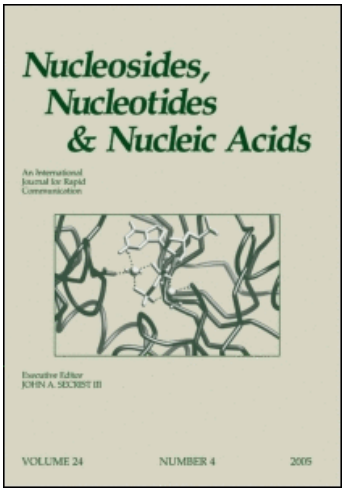

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HISTOLOGICAL LOCALIZATION OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES IN NORMAL RODENT TISSUE

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Abstract: The distribution of phosphorothioate oligodeoxynucleotides (P=S ODN) in rodent tissues was studied *in vivo* using three histological methods: direct fluorescence microscopy; immunohistochemistry; and autoradiography. All three methods gave essentially the same pattern of oligonucleotide localization in the tissues studied, and the histological results correlate well with those from radiochemical and biochemical studies of P=S ODN distribution.

Phosphorothioate oligodeoxynucleotides (P=S ODN) are being developed as therapeutic agents for a wide variety of diseases including cancer and AIDS. The disposition of P=S ODN in animals after intravenous injection has been well characterized using radiochemical and biochemical techniques (1). Such studies show, for example, that P=S ODN are rapidly cleared from the plasma into tissues, and that the liver and kidney accumulate the highest percentage of P=S ODN. However, the uptake of P=S ODN by specific cell types within tissues is less well-characterized. In this study, we sought to better describe the cellular uptake of iv. administered P=S ODN using three different histological techniques: direct fluorescence microscopy of P=S ODN conjugated to rhodamine; immunohistochemistry using monoclonal antibodies against a specific P=S ODN; and autoradiography of ^{14}C-labeled P=S ODN. In order to ensure that P=S ODN remained localized as *in vivo* during histological procedures, the systemic circulation was perfused with fixative prior to the removal of tissues from the experimental animals.

Methods: For immunostaining studies, Sprague-Dawley rats were injected in a tail vein with 5 mg/kg, 20 mg/kg or 50 mg/kg doses of ISIS 2105, a full phosphorothioate oligodeoxynucleotide against human papillomavirus. The animals were anesthetized at 2 h, 24 h, or 72 h post-injection and transcardially perfused with saline, followed by freshly prepared 4% paraformaldehyde and 0.2% glutaraldehyde in PBS. The kidneys, pinnas, sections of liver, lung, spleen, small and large intestine, and knee joints and

associated bones were then removed and post-fixed for 2 h. All the tissues were paraffin embedded, and 4 micron sections were cut and deparaffinized. The sections were then immunostained using a primary mouse monoclonal antibody raised against ISIS 2105, and a secondary peroxidase-conjugated donkey anti-mouse antibody. DAB was used to visualize the peroxidase activity, and hematoxylin was used as a counterstain. Tissues from saline-injected animals were used as controls and treated the same way as tissues from injected animals.

For fluorescence microscopy experiments, Balb/c mice were injected with 5 mg/kg or 20 mg/kg ISIS 2105 conjugated to rhodamine via an aminolink on the 5' end. After 2 h or 24 h, the mice were perfused, and the tissues were removed and treated as above. The tissues were embedded in OCT, and 5 micron cryostat sections were collected on slides and air-dried overnight. An aqueous media was used to mount all slides before viewing under ultraviolet epifluorescence on a Nikon microscope.

For autoradiography studies, Balb/c mice received 5 mg/kg or 20 mg/kg doses of ^{14}C-labeled ISIS 2302, a full phosphorothioate oligodeoxynucleotide against human ICAM, and were perfused at 2 h, 24 h, or 72 h after injection, as described above. Tissues were processed for paraffin embedding, and 4 micron sections were cut and deparaffinized. The slides were then coated with Kodak NTB_2 emulsion and exposed at -80°C. The slides were developed with Kodak D19 developer, followed by Kodak Unifix, and were counterstained with Nuclear Fast Red and Metanil Yellow.

Results and Discussion: The systemic perfusion of animals with fixative effectively trapped P=S ODN within tissues and prevented their diffusion during histological procedures. Immunostaining and autoradiography worked equally well on cryostat or paraffin-embedded sections, indicating that tissues did not lose P=S ODN during the process of paraffin embedding, as other investigators using snap-frozen tissue have reported (2). Furthermore, there was no visible diffusion of fluorescent P=S ODN in sections of perfused tissue with time, and P=S ODN were not extracted from perfused tissues by proteinase K digestion followed by chloroform/phenol extraction.

Immunostaining, fluorescence microscopy, and autoradiography gave essentially the same pattern of oligonucleotide distribution, irrespective of dose, time or tissue. In liver, oligonucleotide was detected in Kupffer cells, endothelial cells, and hepatocytes using all three methods (Fig. 1). In kidney, cells in the glomerular capsule and proximal tubules

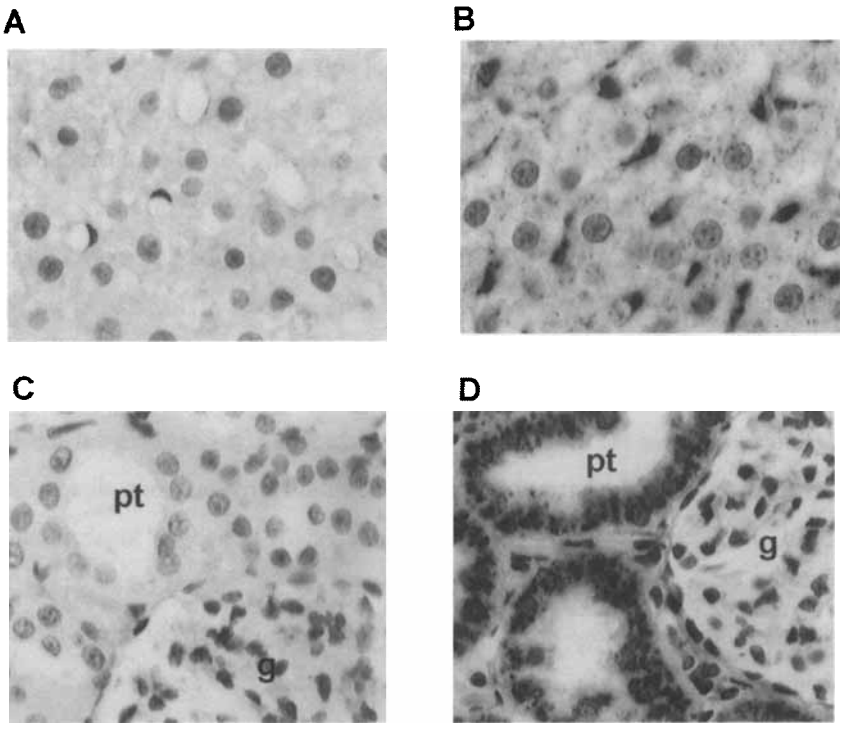

FIG. 1 Immunostaining of rat liver and kidney at 2h after injection of 20 mg/kg of ISIS 2105. *A*, control liver; *B*, ISIS 2105 liver: note staining of Kupffer cells and positive vesicles in hepatocytes. *C*, control kidney; *D*, ISIS 2105 kidney: note heavy staining of proximal tubules (pt) and negative glomeruli (g). Nuclei were counterstained with hematoxylin.

were uniformly and heavily stained, whereas cells lining the loops of Henle, distal tubules, and collecting ducts were usually negative (Fig. 1). Reactive cytoplasmic vesicles could be visualized in many of the labeled cells in liver, kidney, and other tissues by both immunostaining and fluorescence microscopy. Increasing the dose of P=S ODN did not significantly change the pattern of oligonucleotide distribution, but did increase the strength of the histological signal.

The major differences in P=S ODN distribution at 2 h versus 24 h post-injection were observed in connective tissues. Between 15 minutes and two hours post-injection, P=S ODN are detected in the extracellular matrix in many loose and dense connective tissues, such as the lamina propria of the mucosa and in the submucosa of the small

intestine, and the dermis of the skin. By 24 h, most of the oligonucleotide signal was intracellular in the same tissues. The epithelial cell layers in the intestine and skin were predominately negative at all time points. Similarly, the matrix and cells in the connective tissues associated with skeletal muscle were strongly positive for oligonucleotide at early timepoints after dosing, whereas the muscle cells were negative. Also, many large, nucleated cells within red bone marrow were positive for P=S ODN, as were cells in the endosteum lining the marrow cavities. Oligonucleotides were not detected in cartilage, bone matrix, or blood.

In summary, all three histological methods gave essentially the same pattern of P=S ODN localization in the tissues studied. The strength of the histological signal within tissues correlated well with pharmacokinetic data. Of the three methods, direct fluorescence microscopy and immunostaining produced the best signal localization and resolution. Rhodamine conjugation did not appear to significantly effect P=S ODN distribution and thus can be used as a reliable and relatively simple method of determining oligonucleotide localization in cells and tissues.

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