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Methods Review

Skin research and drug localization with receptor microscopic autoradiography

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Abstract: For the localization of drugs and related functional characterization, cellular–subcellular resolution can be achieved with radiolabelled compounds of high specific activity and receptor microscopic autoradiography, which is the method of choice for high-resolution qualitative and quantitative imaging. Detailed information together with integrative surveys can be obtained which is impossible with other methods. The history of discoveries of drug targets testifies to the utility and potential of receptor microscopic autoradiography that was designed to preserve *in vivo* conditions by excluding any liquid treatment during tissue preparation and to avoid translocation and loss of diffusible compounds. Examples of autoradiograms include *in vivo* applications of ^{3}H-vitamin D, ^{3}H-OCT (oxacalcitriol), ^{3}H-oestradiol and ^{3}H-retinoic acid. For topical applications, routes of delivery and sites of deposition and retention are demonstrated.

Key words: autoradiography – drug localization – hair – imaging–keratinocytes – skin penetration – target cells – topical application

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Introduction

For understanding actions of bioactive compounds and therapeutics, knowledge of sites of tissue deposition and cellular receptor binding is necessary. Information can be gained through the use of radiolabelled compounds and their tracing *in vivo*. If cellular and subcellular resolution is to be obtained, receptor microscopic autoradiography (microautoradiography) is the method of choice (1). Other methods in use for drug localization include radioassay, whole-body macroautoradiography and nuclear imaging. The methods, in most cases, do not provide necessary sensitivity and resolution. Even the use of autoradiography requires special precautions and attention to detail to avoid misleading information. Numerous 'expedient' modifications of the autoradiographic approach have been applied and advocated, often compromising the utility of an otherwise powerful technique by providing a confusing mix of results and artifacts.

There are different requirements of method, depending on the type of compound studied. For autoradiography with precursor molecules, like amino acids for protein synthesis, or thymidine for DNA synthesis, as well as with peptides immobilized by fixation, the tissue can be prepared by traditional histological fixation-embedding procedures. With this kind of autoradiography, there is no major threat of leaching and translocation. This review deals with the localization of diffusible compounds, hormones and drugs, that are not covalently bound and would be translocated and lost during liquid fixation, embedding and liquid emulsion dipping.

To trace the delivery, tissue deposition and specific receptor binding of bioactive compounds, radioisotope tagging is a common approach. The label should be in a chemically defined position and must not change the binding affinities of the original molecule. High specific activity labelling engenders high sensitivity and high resolution which is necessary for detecting low-capacity high-specificity receptor sites that otherwise would remain unrecognized. The *in vivo* deposition of the compound must remain unchanged and preserved. For optimal *in vivo* tissue preservation, ideally 'the unmolested tissue' is studied. Cellular and subcellular resolution requires dissection and handling. Here lies the problem. Many methods for 'imaging' have been recommended, striving for authenticity of

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in vivo distribution, high resolution, and, at the same time, expediency and convenience. No single method achieves all. Low-resolution data, e.g. from radioassays and whole-body autoradiography alone, remain incomplete, may lead to overinterpretation, and are ultimately misleading and costly. A detailed discussion of the various procedures is beyond the scope of this article and provided elsewhere (1).

Method

Receptor microscopic autoradiography is applicable for diffusible and non-diffusible compounds. A brief outline of its main steps is given here. Knowledge about handling of radioactivity, cell biology and experience of method is prerequisite to avoid failures and artifacts. Major steps of the method are listed in the diagram below:

The method is discussed in detail in a monograph (1). Its important aspects include the following.

RECEPTOR MICRO-AUTORADIOGRAPHY
Sequence of steps

APPLICATION
of radiolabelled compound
with high specific activity
(including competition)
↓
EXCISION
of multiple tissues
and positioning on holders
↓
FREEZE-MOUNTING
on tissue holders
↓
LIQUID NITROGEN STORAGE
of mounted tissues
↓
CRYO-SECTIONING
of 4 μm (or less) sections
↓
THAW-MOUNTING
on emulsion-coated slides
↓
PHOTOGRAPHIC EXPOSURE
in desiccator boxes
↓
PHOTOGRAPHIC PROCESSING
↓
STAINING with MBBF or MGP
↓
AIR-DRYING and COVER-SLIPPING
↓
EVALUATION
qualitative and quantitative

Radiolabelled compounds of high specific activity and purity, i.e. synthetically labelled with tritium in one or several positions with a specific activity above 40 Ci/mm [1.48 TBq/mm; ideally above 100 Ci/mm (3.7 TBq/mm)] are recommended. Experiments with low specific activity compounds may not inform about low-capacity high-specificity sites of tissue distribution, while perhaps adequate for monitoring main routes of delivery, metabolism and excretion, but are likely to require long exposure times and to result in false negatives, such as misjudgement of a blood–brain barrier.

Freezing of tissue and handling must avoid ice crystal formation and tissue destruction. *Freeze-mounting* in isopentane cooled with liquid nitrogen, storage in liquid nitrogen and sectioning in a cryostat below −20°C (depending on type of tissue and other conditions) are appropriate. Positioning of tissue for freeze-mounting is important to facilitate trimming and sectioning in the right planes and areas.

Frozen sectioning requires a microtome cryostat (e.g. Microm and Leica, both Walldorf, Germany) that enables sectioning of 4 (or 3) μm ribbons. Sectioning is performed under light in a darkroom.

Thaw-mounting on dry emulsion-coated slides is performed under safety light (e.g. Kodak OC filter, Eastman Kodak, Rochester, NY, USA). Proper thaw-mounting without pressure and without refreezing is crucial to avoid related artifacts. Practice and controls are required.

Exposure of mounted slides in lightproof desiccator boxes may be done in a freezer or refrigerator for different lengths of time. The exposure time cannot be calculated in advance but has to be found empirically, as the specific concentration in the tissue is unknown and assessed through the experiment. Different exposure times provide different information, which is important for the interpretation of data.

Development and staining should be carried out with precaution. If not performed correctly, in each step artifacts can be introduced. For instance, a black box after removal from cold storage should be opened only after its content has fully assumed room temperature, otherwise precipitation of moisture and latent image fading may ensue. A single-step stain with methylgreen-pyronin (MGP) for DNA–RNA or methylene blue-basic fuchsin (MBBF) provides beautiful distinction of structure, superior to the traditional H&E stain.

Controls against false positives, e.g. without radioactivity or 0-day exposure of radioactive samples, should be included. For avoiding false negatives and for low-density quantification and high-density low-magnification surveys, long and short exposure times may be considered.

Autoradiography combined with immunocytochemistry can be applied for the characterization of target tissues.

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Its general feasibility has been demonstrated with neurons, pituitary cells, heart atrial myocytes and stomach gastrin cells (1). Immunocytochemistry does not yield the same information as microautoradiography. While useful for the identification of proteins, immunocytochemistry with antibodies to receptors or drugs cannot provide data on drug localization, neither qualitative nor quantitative, unless compounds are covalently bonded to protein.

Introductory experiment

A practice run is recommended with a compound known to be localized at specific sites. Thus, errors in the procedure and related artifacts can be recognized, true results distinguished from artifacts and future pitfalls avoided. For an introductory, inexpensive and simple experiment, ^{3}H-thymidine may be used. After intraperitoneal injection of 1 or 2 μCi/g bodyweight and killing after 1 h, procedures of freeze-mounting of excised tissues, frozen sectioning and thaw-mounting on emulsion-coated slides can be practiced. Cells in S-phase of the cell-cycle display nuclear labelling after 2–4 weeks of exposure time. The importance of proper orientation of tissue blocks, of section thickness, staining, etc. becomes apparent (1).

It needs to be emphasized that receptor microscopic autoradiography is the tested method of choice for the topographic tissue localization of diffusible compounds, applicable to any other compound. Assiduous adherence to the protocol is recommended. Quantification of developed autoradiograms by silver grain counting is facilitated through commercially available computer programs.

Specific considerations for skin

In the experimental design, different regions of skin may be considered, also different doses, time intervals and exposure times. Comparisons of skin results with those from other tissues can be informative. Skin autoradiograms have been more difficult to prepare compared with other soft tissues, requiring special considerations. For skin, a proneness of artifacts has been observed, probably related to the presence of sulfhydril groups in the ground substance that can interact with photographic emulsion, and variable densities of collagen, keratin, and hair. Chemical and mechanical artifacts easily ensue, especially if sections thicker than 4 μm are thaw-mounted and inappropriate pressure is used. Such artifacts are apparent by their variability among different sections of the same tissue. Care must be taken not to damage with forceps or scissors tissue samples during excision and positioning onto tissue holders.

For freeze-mounting, the excised strip of skin can be kept flat or rolled with one end inserted into the minced liver base on the metal holder. Proper orientation facilitates sectioning of hair, papillae, and epidermis in the desired plane and level for optimal evaluation and pictorial demonstration of the developed autoradiogram.

After *topical application*, regions of skin at the application site as well as at various remote sites may be excised and studied to assess diffusion and transportation of radiolabelled compound and define its range of action. Internal target and non-target tissues may be included (e.g. in the case of vitamin D, samples of intestine and kidney) to assess the degree of blood penetration and related systemic sites of depositions with possible remote effects.

Results

Systemic application

Examples are provided in Figs 1 and 2 with tritium-labelled vitamin D, OCT, oestradiol and retinoic acid. Changes of receptor binding during neonatal development of hair are depicted in Fig. 1a–d. Hair pegs originate with strong vitamin D nuclear receptor binding in a few keratinocytes in the stratum spinosum. When growing into the dermis, the

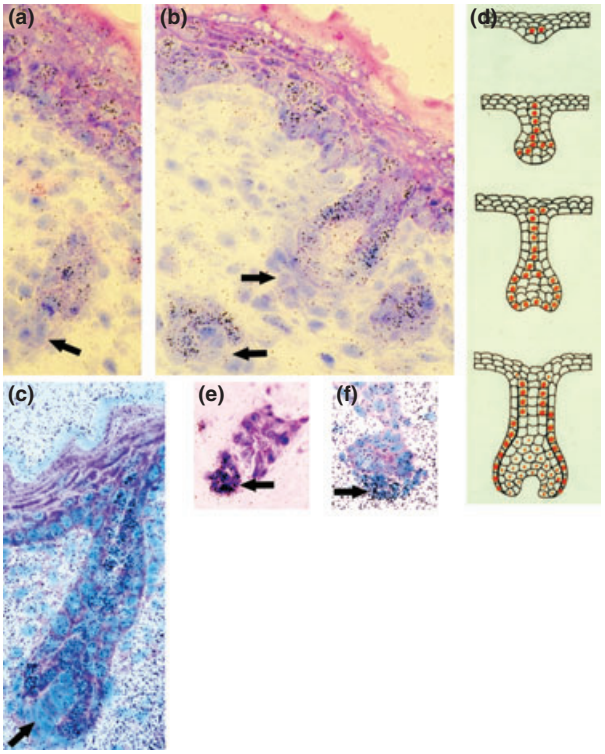

Figure 1. Autoradiograms with ^{3}H-1,25(OH)$_2$ vitamin D$_3$ (a–c) in neonatal rats (3) and ^{3}H-oestradiol (e) (2) and ^{3}H-retinoic acid (f) (19) in mice. With ^{3}H-vitamin D during hair development, select and differential nuclear receptor binding in keratinocytes (a–c), initially in the stratum spinosum, then in the outer hair sheath (schematic in d, red dots represent strongest radiolabelled keratinocytes) (3,21). In dermal papillae (arrows), nuclear labelling is absent or low with vitamin D, but strong with oestradiol (e) and retinoic acid (f).

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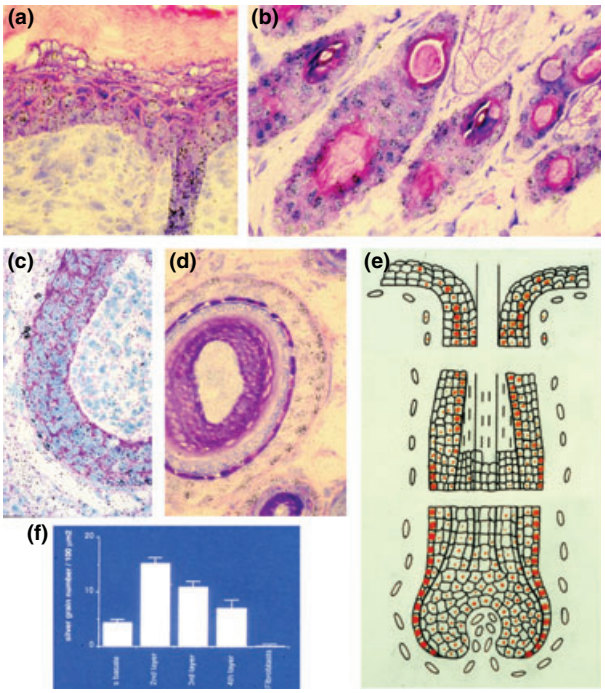

Figure 2. Autoradiograms with ^{3}H-1,25 $(OH)_2$vitamin D_3 (a–c) and its analogue ^{3}H-OCT (d), showing strong nuclear labelling in hair sheath of vellus hair (a, b) and in outer hair sheath of terminal hair (c, d). Nuclear silver grain counts (f) of keratinocytes in cell layers of the epidermis and of fibroblasts in the dermis, suggesting strongest effect on cells of the stratum spinosum, but little on fibroblasts (3). The schematic (e) summarizes the results from autoradiograms with similar distribution of vitamin D and OCT, showing changes of nuclear receptor binding (red dots) along the course of a terminal hair (21).

most strongly labelled keratinocytes are shifting to the outer hair sheath (3,4). There is little or no nuclear receptor binding of vitamin D to cells of dermal papillae. The latter is in contrast to oestradiol and retinoic acid, for which strong nuclear concentration is apparent in cells of dermal papillae (Fig. 1e,f). With vitamin D, connective tissue cells in the dermis in general are without or with occasionally low nuclear concentration. Oestradiol, in contrast to vitamin D, concentrates strongly in fibroblasts of the dermis (2).

Figure 2 provides examples of adult epidermis and hair. The differential distribution of nuclear concentration can be assessed quantitatively as shown in Fig. 2f (3), suggesting predominant actions in the stratum Malpighii on cell differentiation and proliferation. Keratinocytes in hair sheaths concentrate and retain vitamin D. In vibrissae, this is most conspicuous in the outer hair sheath. A differential nuclear receptor binding exists along different regions of hair (Fig. 2e schematic). The connective tissue sheath of hair reveals no or very low nuclear binding, In the region of the infundibulum of vibrissae (Fig. 2e), weak nuclear binding in fibroblasts can be recognized.

Topical application

Examples of percutaneous absorption in Fig. 3 document the two penetration routes through hair canals and diffusion via epidermis, with concentration gradients in the dermis. High concentration of radiolabelled steroid exists in the stratum corneum and in insterstitial spaces of the stratum granulosum. The strong concentration and retention in the stratum corneum suggests a barrier, retention and slow release. Viewed with cellular–subcellular resolution (Fig. 3d), in cells of the stratum granulosum, spinosum and basale, a cytoplasmic concentration of labelled hormone is apparent, while the interstitial spaces in the stratum spinosum and basale are low or devoid of radiolabelled compound. This epidermal high-dose distribution

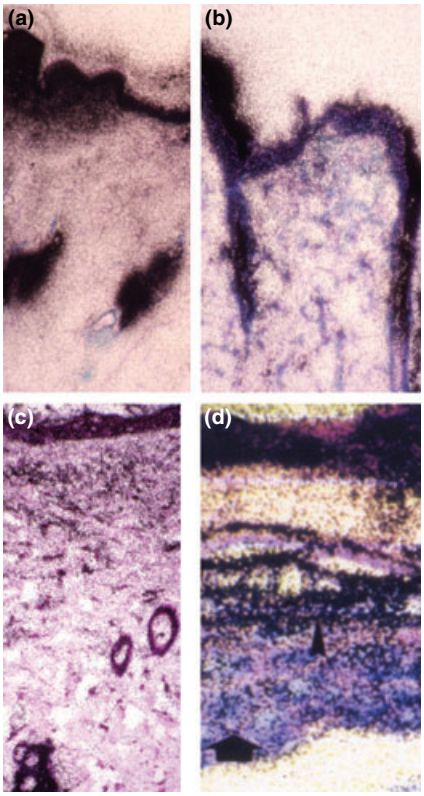

Figure 3. Autoradiograms of skin penetration after topical *in vivo* application of ^{3}H-oestradiol (a–c) (22) and ^{3}H-1,25$(OH)_2$vitamin D_3 (d) (5), demonstrating two routes through layers of the epidermis and hair canals. High magnification (d) shows retention of hormone in the stratum corneum and in interstitial material in the stratum granulosum (small arrow) as well as high cytoplasmic concentration but low or no nuclear concentration in cells of the stratum spinosum and basale (large arrow). By contrast, after systemic (Figs 1 and 2) or low dose topical application, concentration of labelled compound is high in nuclei and low or absent in cytoplasm. These differences in subcellular distribution correlate with the enantiodromic reversal (6) of vitamin D stimulation to inhibition of cell proliferation-differentiation as applied for the treatment of wound healing versus that of psoriasis. Retention of radiolabelled compound in the stratum corneum and granulosum-interstitium suggests functions as a barrier and a depot for slow release.

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pattern of vitamin D differs from that seen at low doses. These dose-related differences in hormone localization correlate with the enantiodromic effects of high-dose versus low-dose treatment (5,6).

Discussion

Evidence indicates that receptor microscopic autoradiography is of considerable diagnostic value because of its high resolution and sensitivity. With this method, the localization and characterization of hormone and drug targets is achieved and information on target pharmacokinetics obtained (7). A combination with immunocytochemistry has been used to simultaneously demonstrate the presence of radioligands and receptors or other specific proteins (8). Immunocytochemical characterization of receptors has been pursued (9,10). However, immunocytochemistry is not useful for drug localization, unless drugs and their metabolites can be immobilized through fixation at their original *in vivo* sites of deposition. Small molecular weight drugs and hormones are not covalently bonded to receptors and are diffusible. Any fluid treatment of tissue may incur translocation and leaching. With immunocytochemistry, precise quantification is also not possible. Furthermore, problems of antibody specificity, epitope recognition, and modifying effects of fixatives complicate and sometimes compromise its utility. While there is assumed correspondence between the immunocytochemical localization of receptor protein and related ligand binding, sites of receptor protein and ligand binding are not always congruent, e.g. as indicated in experiments with oestradiol and especially vitamin D (11). Reasons for that relate to different method sensitivities and/or different receptor affinities, a topic which requires further comparative studies. In general, autoradiography and immunocytochemistry provide different and complimentary information (12). A detailed discussion is beyond the scope of this review.

The use of radioactivity for localizing drugs is perceived as a hindrance by some. Radioactive tagging yields high sensitivity and resolution. Therefore, the use of radiotracers will remain state of the art. Microscopic autoradiography utilizes this potential. The combination with genetically tagged (e.g. green fluorescent protein) receptor proteins and other gene products of transgenic animals (13–16) may open a vast area of new applications for the study of hormone-like substances and their induced cellular responses and dynamics. With high-resolution autoradiography, leads for biochemical and clinical follow-up can be provided (17,18), and results from expedient low-resolution approaches and from *in vitro* studies can be validated and complemented. The execution of receptor microautoradiography, similar to any method with high sensitivity, demands careful attention to detail. Conditions of the labelled compound must be observed regarding specific activity and purity. Tritium, followed by ^{125}I (with some restrictions), provides optimal resolution. Digressions from the time-tested method of receptor micoautoradiography may compromise quality through creating hidden artifacts mixed with true data. A meticulous pursuit will produce both highly informative and beautiful results.

Topical applications

After topical application, percutaneous absorption and penetration of radiolabelled compound can be monitored. (5,20,22). The utility of results obtained with *in vitro* systems, like the Franz static and Bronaugh flow-through diffusion cell system, can be assessed and validated through comparisons with results from autoradiography. With receptor microautoradiography, important information has been gleaned in time- and dose-related penetration studies. For instance, with vitamin D, a sustained concentration and retention (depot formation) has been demonstrated in the stratum corneum and in interstitial spaces in the stratum granulosum. Exposed to a high dose of radiolabelled steroid, keratinocytes in the stratum granulosum, spinosum and basale show high concentration of radiolabelled compound in cytoplasm, while radiolabelled compound is low or absent in nuclei (Fig. 3d). This is contrary to the characteristic high nuclear concentration and low cytoplasmic presence after a low systemic or topical dose. These differences in subcellular binding suggest relationships to different modes of action linked to low-dose positive (wound-healing) and high-dose negative (psoriasis) effects on cell proliferation (8). Such findings can provide an important basis for experimental and clinical follow-up studies.

Multiple factors that govern hair growth

Receptor microscopic autoradiography can help clarify the complex actions of hormones and drugs that influence hair growth. Changing interactions among multiple hormones and structures involve oestrogen, androgen, retinoic acid, vitamin D, thyroid hormone, cortisol and several others, with specific actions on dermal papillae, keratinocytes in hair bulbs, in outer and inner hair sheaths, in developing hair pegs, and on connective tissue sheaths. Therapy of hair loss has been attempted with a single compound, for instance, antiandrogen. Such focused strategies appear inadequate and invite failure in view of the well-known involvement of multiple hormones in the growth and maintenance of functions of skin appendices. During the hair cycle, with microautoradiography specific sites of time-related actions of hormones can be revealed and both qualitative and quantitative information on receptor binding gained that can serve as a basis for a rational therapeutic approach.

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Imaging for drug development and target pharmacokinetics remains a challenge in dermatology. Results from methods without cellular resolution are unsatisfactory. High-resolution receptor-drug diagnostic imaging is possible with receptor microscopic autoradiography. Other modern technologies, e.g. such based on mass spectrometry imaging and matrix-assisted laser desorption ionization, if further developed, may contribute.

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