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Eur J Cancer, Vol. 27, No. 6, pp. 785–789, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00
Pergamon Press plc

Morphological Studies Using *in situ* Hybridisation

Giorgio Terenghi and Julia M. Polak

INTRODUCTION

THE UNDERSTANDING of cellular processes requires investigation of translated peptides and proteins, and of their mRNA transcription. Immunohistochemistry is a powerful tool in identifying peptide storage in well defined anatomical structures, and it has been used successfully in a variety of investigative and diagnostic situations [1, 2]. Detection of mRNA can be achieved using northern blot analysis on tissue extracts. However, this technique has certain limitations, particularly in the analysis of heterogeneous tissues with mixed cellular populations, where a dilution effect of the mRNA under investigation might offset the sensitivity of the technique.

The recent development of reliable *in situ* hybridisation methodologies has allowed the identification of mRNA to specific cell types. This technique is based on the use of labelled nucleic acid probes which are able to link with complementary RNA or DNA target sequences to form a hybrid molecule. Because of the specificity of complementary base pairing between nucleic acids, *in situ* hybridisation allows to identify specific gene expression with anatomical accuracy.

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Received 11 Mar. 1991; accepted 18 Mar. 1991.

TISSUE PREPARATION

To obtain optimal results with *in situ* hybridisation it is necessary to achieve nucleic acid retention combined with preservation of tissue morphology; hence the use of appropriate fixation is indispensable. Different authors favour a variety of fixatives, and several studies have tried to demonstrate that a specific fixative might be better than others [3–6]. Although the final choice is dictated by the type of tissue and the method of processing, paraformaldehyde appears to be the most widely used reagent for peptide mRNA detection.

The rate of mRNA degradation varies considerably for different sequences, and endogenous nucleases, particularly RNase, appear to be the major contributors in the degradation process. Because the mRNA breakdown is generally rapid, it is recommended to carry out the fixation with a minimum delay. A 10–20 minutes delay from tissue collection is considered acceptable as longer delays can have an adverse effect on hybridisation results [7]. Interestingly, successful results for *in situ* hybridisation have been seen on tissue collected up to 10 hours postmortem [8, 9]. This might be explained by a slow degradation process in cooled untouched tissues [10, 11], as it has been observed that dehydration, manipulation and cutting of unfixed samples during routine dissection accelerate the degradation process, most probably by release of lysosomal content and of endonucleases [10].

PROBES

The probes used for *in situ* hybridisation are of three types: DNA probes, RNA probes and oligonucleotide probes. Double stranded DNA probes were the first to be used for *in situ* hybridisation, and although favoured by several authors they present certain disadvantages. They need to be denatured to enable complementary single strands to hybridise with the target sequence, but the reannealing of the DNA strands can in effect decrease the amount of probe available for hybridisation [12]. Also their large size (typically about 1000 base pairs or over) might preclude an efficient penetration into the tissue.

At present RNA probes [12] are used for most of our work, as they offer a number of advantages. RNA probes are easily synthesised using RNA polymerase [13, 14]. They are single stranded sequences of predetermined and constant size, and do not require denaturation prior to use. As reannealing in solution does not occur, all the probe is available for hybridisation. RNA/RNA hybrids are more stable than DNA/RNA ones [12], hence higher stringencies can be used during hybridisation and washes to prevent and remove non-specific probe binding. Incorporation of radioactively labelled nucleotides is high (up to 80%) and the resulting probes have high specific activity and sensitivity of detection. Alternatively biotinylated [15] or digoxigenin-labelled nucleotides [16] can be used to obtain non-radioactively labelled probes.

Oligonucleotide probes can be easily synthesised if the target sequence is known. They are small in size (typically 30–50 bases), which is advantageous for probe penetration, and are single stranded DNA sequences, but the available labelling methods allow the incorporation of a small number of tagged nucleotides, which reduces considerably the sensitivity of detection. More recently it has been possible to synthesise oligonucleotides complete with a promoter sequence for RNA polymerase, which allows to transcribe uniformly labelled RNA probes from an oligonucleotide template [17].

CHOICE OF LABEL

The labelling methods vary according to the type of probe [6]. However, the choice of label depends on several factors such as resolution, sensitivity, rapidity of results, quantification, cost and safety. At present none of the available labels satisfies equally well all these criteria, but certainly the need for sensitivity and resolution plays a major part in the final choice.

Radioactive labels are considered to be the most sensitive, although resolution and rapidity do not always coincide when using different isotopes [18]. Labelling with ^{32}P produces high specific activity probes, hence allowing rapid (1–2 days) and sensitive detection. However, the resolution is generally poor because of the silver grain scatter on the microautoradiograms created by high energy β particles, and ^{32}P-labelled probes are best suited for rapid screening and film autoradiography.

In contrast ^{3}H-labelled probes allow a precise resolution at the cellular level, but the autoradiographic exposure time is generally very long, with a minimum of 3–4 weeks when target sequences are abundant. A shortening of the exposure time can be achieved by incorporating more than one ^{3}H-labelled nucleotide into a probe [6]. ^{35}S is a suitable compromise between the previous two isotopes, as it gives good resolution within relatively short autoradiography exposure time (5–7 days). The problem of background commonly encountered with this label can be solved by using high concentration of dithiothreitol (DTT) in hybridisation buffer and stringency washes [4]. Furthermore, ^{35}S-labelled probes can be used for densitometric analysis on film autoradiograms [19].

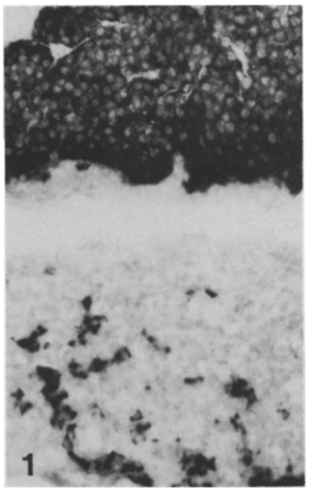

Fig. 1. Section of rat pituitary hybridised with digoxigenin-labelled pro-opiomelanocortin probe. Strong hybridisation signal is seen in all cells of the intermediate portion and in scattered cells of the anterior portion of the pituitary. The hybrids were detected using anti-digoxigenin antibodies conjugated with alkaline phosphatase.

Several methods for labelling probes with non-radioactive labels have been published [20–22], but the most commonly used labels appear to be biotin [23] and digoxigenin [16, 24]. Initially biotin was used mainly for labelling DNA probes, but more recent reports have shown successful results with biotinylated RNA [15, 25] and oligonucleotide [5, 26] probes. Digoxigenin-labelled probes have been introduced only recently, but they have already gained widespread favour as they show an increased sensitivity compared to biotinylated probes [27, 28], and avoid the background problem generally associated with endogenous biotin (Fig. 1). When digoxigenin and biotinylated-labelled probes are used simultaneously, it is possible to identify two separate target sequences with a single *in situ* hybridisation procedure [24]. Although in the past non-radioactive labelled probes have been criticised for the low level of sensitivity, recent publications have shown a sensitivity comparable to that of radiolabelled probes [28–30]. Also, it has been shown that the choice of detection system can greatly influence the level of detection and resolution of final hybridisation results [15].

STUDIES OF THE NEUROENDOCRINE SYSTEM

We have used *in situ* hybridisation mainly to study the diffuse neuroendocrine system, but the technique can be equally applied to all aspects of morphological and pathological investigations. Regulatory peptides have been identified in both normal and pathological tissues by immunohistochemistry [1, 2]. By using *in situ* hybridisation the site of synthesis of many of these peptides have now been confirmed beyond any doubt [7, 8, 31, 32]. In some cases *in situ* hybridisation has given more complete insight into the cellular metabolism of specific peptides.

In pathology *in situ* hybridisation has been widely used [33], particularly to investigate endocrine tumours, as often characterisation by peptide immunohistochemistry cannot be carried out because of peptide hypersecretion and limited storage capacity of the cells. For example, gut carcinoid tumours can be better characterised by the detection of tachykinin gene expression in

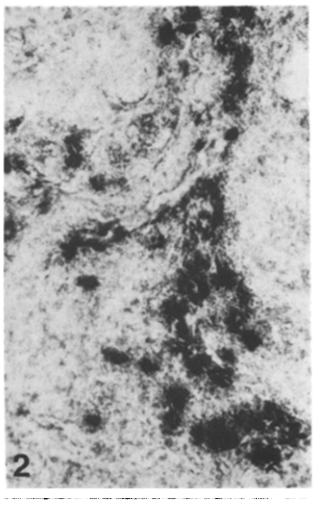

Fig. 2. Section of human small cell carcinoma of the lung hybridised with ^{32}P-labelled GRP probe. The hybrid was visualised by emulsion microautoradiography.

tumour cells [34]. The small cell carcinoma of the lung often secretes gastrin-releasing peptide (GRP), although immunoreactivity for it is difficult to demonstrate in the tumour cells. However, GRP mRNA was easily demonstrated by *in situ* hybridisation in numerous cells in all studied tumours (Fig. 2) [35]. Endothelin-1 (ET-1), a novel peptide first identified in endothelial cells [36], has been localised to pulmonary endocrine cells and in bronchial epithelium, and a role of this peptide in promoting cellular growth has been suggested. The distribution and possible role prompted the investigation of endothelin expression in pulmonary tumours. Interestingly, ET-1 immunoreactivity and mRNA were found in squamous cell carcinomas and adenocarcinomas but not in small and large cell carcinomas [37]. The results of this and previous studies tend to suggest that the tropic effect of ET-1 and GRP might be directed to different cell types.

Chromogranin A (CgA) is a protein shown by immunohistochemistry to be specific for both normal and malignant cells of the neuroendocrine system. High levels of CgA are found in many patients with neuroendocrine tumours, but CgA immunoreactivity can not always be detected in small cell carcinoma of the lung, possibly owing to poor granularity. Using *in situ* hybridisation it was possible to show CgA gene expression in all small cell carcinoma investigated, but not in non-endocrine lung tumour [38], confirming the specificity of this marker for neuroendocrine tumours. Oncogenes have also been widely investigated by *in situ* hybridisation, and several reports have now shown that oncogene expression can be related to tumour progression [39, 40], particularly in lung small cell carcinoma [41] and neuroblastoma [42, 43].

Calcitonin gene-related peptide (CGRP) is a neuropeptide abundantly distributed in primary sensory neurons. Unexpectedly, CGRP immunoreactivity was also found in motoneurons in ventral horn of the spinal cord. Because of the sensory related functions attributed to the CGRP, the origin of the staining in motoneurons was questioned and the suggestion was put forward that it might be due to sensory neuronal terminals synapsing onto the motoneurons. By applying *in situ* hybridisation it was possible to determine that CGRP synthesis was present in both sensory and motoneurons [9]. Primary sensory neurons show immunoreactivity for many other peptides, and it was also possible to co-localise mRNA for CGRP, substance P and ET-1 in well defined subpopulations of neuronal cells in dorsal root ganglia [44].

A different type of problem can be encountered when studying the developmental pattern of neuropeptides. By 8 weeks gestational age, in human fetal gut it was possible to detect a considerable network of neuronal cells and fibres immunoreactive for protein gene product 9.5 (PGP), a pan-neuronal marker. At the same time vasoactive intestinal polypeptide (VIP) immunoreactivity was very sparse and confined to few fibres innervating the myenteric plexus, whose origin could not be determined. VIP-immunoreactive neuronal cells could not be detected until 18 weeks gestation, while *in situ* hybridisation allowed the detection of VIP mRNA in ganglion cells of the gut at 9 weeks gestation [45]. The detection of neuronal cells by *in situ* hybridisation at much earlier gestational age than by immunohistochemistry also allowed to follow the neural migration and colonisation of the gut, which showed a transmural gradient and craniocaudal pattern of maturation [45].

IN SITU HYBRIDISATION AND IMMUNOHISTOCHEMISTRY

The combined application of these two techniques offers a suitable complement for morphological investigation of peptide synthesis and storage. *In situ* hybridisation and immunohistochemistry can be carried out on separate or serial sections [8, 31, 46], but maximum advantage can be obtained by their sequential application on the same section, particularly when using an homogeneous tissue with few recognisable landmarks [19, 44, 47, 48].

The combined use of the two methods was particularly advantageous in functional studies on the pituitary. The number of cells immunoreactive for prolactin and the intensity of immunostaining appeared very similar in control pituitary and in animals during pregnancy and lactation, despite a well known variation of circulating peptide in these physiological conditions. When *in situ* hybridisation was carried out in combination with immunohistochemistry, the pituitary cells showed an evident decrease of prolactin synthesis during pregnancy and an increase during lactation [46], clearly showing a variable degree of peptide expression, storage and release during different functional states.

QUANTIFICATION OF *IN SITU* HYBRIDISATION

Sometimes it is important to assess accurately and objectively the changes observed by *in situ* hybridisation. At present radiolabelled probes are favoured when quantification is required, as they offer a choice between methods of assessments. Silver grain counts following microautoradiography is one of the methods being used for quantification of the hybrids [49]. This method offers the advantage of single cell resolution, but it is important to be aware of the possible problems due to autoradiography overexposure and silver grain clusters. It is also important to optimise the tissue processing and hybridisation procedure in order to determine a standard curve for correlating grain counts with degree of hybridisation [50]. Because the number of variable in both hybridisation and autoradiography procedures, it is still difficult to determine absolute measurements directly relating to copy numbers of target nucleic acid, and preference is given to relative assessment of experimental samples in comparison to controls.

An alternative method is densitometric analysis which can be carried out on autoradiography films exposed to hybridised

section or cell cultures, and which are compared to radioactive calibrated standards [19, 51, 52]. The labelling of choice in this quantification method was ^{35}S, as the labelling densities can be compared to those of commercially available ^{14}C standards [53]. The effect of endocrine manipulation on mRNA synthesis of beta thyroid-stimulating hormone (β-TSH) was quantified on pituitaries of euthyroid and hypothyroid rats. Densitometric scanning of autoradiograms was carried out by computerised image analysis. The optical densities measured on the tissue autoradiograms was referred to a calibration curve and converted into concentration of radioactivity per unit area [19]. This method allowed to determine precisely that specific labelling for β-TSH in pituitary of hypothyroid rats was 22-fold greater than in control animals.

CONCLUSIONS

It is evident that *in situ* hybridisation can supply information which it is not possible to obtain with other investigative techniques. Hence by combining the use of *in situ* hybridisation with other methodologies it is possible to obtain a more complete insight of cellular metabolic processes in different pathophysiological situations.

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Eur J Cancer, Vol. 27, No. 6, pp. 789–795, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Meeting Report: First Meeting of the "Task Force Cytokines" of the EORTC Research Branch

H. Zwierzina

THE RESEARCH BRANCH (Chairman: M. Rajewsky) of the EORTC was founded to narrow the gap between basic scientists and clinicians. A very promising field for cooperation between these groups is represented by cytokines, because these factors have already been introduced into *in vivo* clinical trials while, on the other hand, much scientific work remains to be done toward understanding the mechanisms of action underlying this promising new biological therapy.

Cytokines are produced by a large number of cells and serve to transmit signals mainly within the haemopoietic and the immune systems either by themselves or by inducing the production and release of other cytokines. These factors enable clinicians to carry out completely new therapy concepts by allowing them to intervene in and modulate physiological processes. The term "cytokine" includes more or less well known factors such as interferons, tumour necrosis factors (TNFs), haemopoietic growth factors and many others such as transforming growth factors (TFG-α, TGF-β), platelet-derived growth factor (PDGF) or epidermal growth factor (EGF). For historical reasons, all of these maintain their names, although these often do not correspond to their actions. Since the 6th International Congress on Immunology in 1986, every newly discovered human cytokine has been called "interleukin" (IL) once the aminoacid sequence has been defined. The number of interleukins is steadily increasing and, at the moment, 11 of these factors are cloned.

Therapy studies conducted to date with cytokines such as growth factors or IL-2 have shown that only limited conclusions can be drawn from *in vitro* and animal experiments since the intact human immune system appears to be required. Thus, new research concepts must be developed and close cooperation between clinicians and basic scientists is essential to understand the complex mechanisms of the cytokine network within the human body and the multiple aspects of a potential *in vivo* application of these factors. For this reason, the Task Force Cytokines of the EORTC Research Branch was founded and a first meeting was held at the Institute of Cell Biology (Cancer Research) in Essen, 23–24 November 1990. This meeting brought together distinguished scientists and clinicians not only from the EORTC but also from other European groups involved in cancer research, who presented their contributions in the field of cytokines and discussed future strategies for cooperation.

INTERFERONS

O. Kloke (Essen) reported on *in vitro* and *in vivo* investigations with interferon-α (IFN-α) and interferon-γ (IFN-γ) in chronic myeloid leukaemia (CML).

IFN-α is a very efficient drug in the stable phase of CML. When Philadelphia chromosome (Ph) positive CML patients are treated with IFN-α in combination with IFN-γ, neither in

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Received 18 Feb. 1991; accepted 4 Mar. 1991.