Melatonin receptors in the human fetal kidney: 2-[^{125}I]iodomelatonin binding sites correlated with expression of Mel1a and Mel1b receptor genes

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Abstract

Melatonin receptors in the human fetal kidney were identified and characterized by quantitative *in vitro* autoradiography using the melatonin agonist, 2-[^{125}I]iodomelatonin. Specific binding was localized to cells in the nephrogenic region at the outer perimeter of the developing kidney and was time-dependent, saturable and inhibited in the presence of guanosine 5′-0-(3-thiotriphosphate) indicative of a G protein-coupled receptor. Expression of the Mel1a and Mel1b melatonin receptors in human fetal kidney was determined using RT–PCR. *In situ* hybridization confirmed the localization of the Mel1a mRNA transcripts. A role for melatonin in development of the human fetal kidney is postulated.

Journal of Endocrinology (1998) **156,** 261–267

Introduction

The role of the pineal hormone, melatonin, in seasonality and circadian rhythmicity has been well documented (for reviews see Reiter 1991, Morgan *et al.* 1994). Melatonin receptors have been localized in the hypothalamus and the pituitary of many species confirming a central site of action for melatonin in these processes (Vanecek *et al.* 1987, Morgan *et al.* 1989, Weaver *et al.* 1993, Reppert *et al.* 1994). However, based on the identification of putative melatonin binding sites in peripheral tissues such as the intestine (Pointoire *et al.* 1993), cerebral arteries (Viswanathan *et al.* 1993) and kidney (Song *et al.* 1993, 1995*a*,*b*, 1997) it is now thought that melatonin may have a potentially wider role than the control of seasonal and circadian rhythmicity. Furthermore, the identification of 2-[^{125}I]iodomelatonin binding sites in the fetuses of a number of different species, including the rat (Williams *et al.* 1991), sheep (Helliwell & Williams 1994), hamster (Weaver *et al.* 1988, Carlson *et al.* 1991, Rivkees & Reppert 1992) and the human (Yuan *et al.* 1991), is indicative of a further function for melatonin in fetal development. Maternal melatonin has been shown to be transferred to the fetus in sheep (Yellon & Longo 1988), hamsters (Weaver *et al.* 1988), rats (Klein 1972) and non-human primates (Reppert *et al.* 1979) and to influence hormonal status in the fetus and in the neonate (Bassett *et al.* 1988, 1989, Ebling *et al.* 1989).

To investigate the potential role of melatonin in the developing human fetus we have localized and characterized specific melatonin receptors in the fetal human kidney utilizing a combination of quantitative *in vitro* autoradiography and *in situ* hybridization using specific riboprobes derived from cDNAs for the two known human G protein-coupled melatonin receptors, designated Mel1a and Mel1b (Reppert *et al.* 1994, 1995).

Materials and Methods

Tissue preparation

Tissue was collected following termination of pregnancy, with the permission of the local ethical committee after informed consent had been obtained. Tissue was frozen immediately in isopentane chilled over dry ice for *in vitro* autoradiography or in liquid nitrogen for mRNA extraction and *in situ* hybridization. Tissues were stored at −80 °C until use.

2-[^{125}I]Iodomelatonin in vitro *autoradiography*

Cryostat sections (20 µm thick) of fetal kidney (16·5, 17·5 and 20 weeks gestation) were thaw-mounted onto gelatin-coated slides. To localize specific 2-[^{125}I]iodomelatonin binding, slides were preincubated for 15 min in 25 mM Tris/HCl buffer containing 4 mM $CaCl_2$ then incubated

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with approximately 80 pM 2-[^{125}I]iodomelatonin (NEN Dupont Ltd, Stevenage, Herts, UK) in the presence or absence of 10^{-7} M melatonin, for 3 h in the same buffer at 22 °C. The saturability of specific binding was assessed by incubating sections with increasing concentrations of 2-[^{125}I]iodomelatonin between 10 and 150 pM. Time dependency and stability of 2-[^{125}I]iodomelatonin binding was verified by incubating serial sections for increasing lengths of time between 15 min and 4 h. The sensitivity of 2-[^{125}I]iodomelatonin to guanine nucleotides was assessed by incubating serial sections in the presence of 10^{-3}–10^{-11} M ATP or 10^{-4}–10^{-12} M guanosine 5′-O-(3-thiotriphosphate) (GTPγS). After incubation, sections were washed in ice-cold buffer (3 × 5 min), briefly rinsed in ice-cold distilled H_2O, air-dried and apposed to Kodak AR X-OMAT film along with 20 µm [^{125}I] polymer standards (Amersham International, Amersham, Bucks, UK). Localization of specific 2-[^{125}I]iodomelatonin binding was initially assessed visually and then quantified using a Torch Quad X Work Station (Torch Computers, Cambridge, UK) according to Nazarali *et al.* (1990). Saturation data and competition curves were analyzed using Grafit (Sigma International, Poole, Dorset, UK). Sections were stained in toluidine blue to facilitate identification of labeled areas or coated in LM-1 liquid emulsion (Amersham International) for light microscope autoradiography.

RT–PCR

Messenger RNA was isolated from a fetal kidney (20 weeks gestation) using a message maker mRNA isolation kit (R & D Systems, Abingdon, UK) according to the manufacturer's instructions. The mRNA was quantified by a GeneQuant II spectrophotometer (Pharmacia Biotech, St Albans, Herts, UK) and an aliquot was electrophoresed through a formaldehyde gel according to the method described by Miller (1987) to determine quality. First strand cDNA was synthesized from 1 µg isolated mRNA in 1 × transcription buffer using 0·5 µg random primers and 200 U Moloney murine leukemia virus reverse transcriptase (RT) in the presence of 0·8 mM dithiothreitol (DTT), 25 U RNA guard and 1 mM dNTPs. PCR was performed on 2 µl aliquots of the 20 µl first strand cDNA reaction using Mel1a or Mel1b specific oligo primers (see Fig. 1). The primer pairs P1MEL1A and P2MEL1A, P1MEL1B and P2MEL1B, P1MEL1B and P3MEL1B were designed to give amplification between exon 1 and 2 of the melatonin receptor gene thus eliminating PCR of genomic DNA (see Fig. 1). Hot start PCR was performed using 100 pmol of each primer, 2·5 U AmpliTaq in the presence of 200 µM dNTPs and 1·5 mM $MgCl_2$. The PCR cycles consisted of 94 °C for 1 min, 58 °C for 1·5 min and 72 °C for 1·5 min for 35 cycles. Control reactions were performed with 1 µg fetal kidney mRNA as above after RNase or DNase treatment.

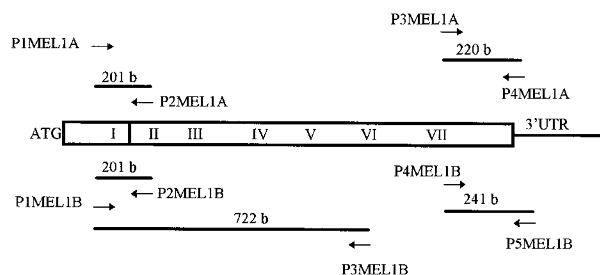

Figure 1 Schematic diagram of melatonin receptor cDNA showing location of primer sites used for RT–PCR and construction of riboprobe templates. The primer pairs shown above the diagram of the melatonin receptor cDNA are specific for the human Mel1a receptor and consist of P1MEL1A (5′-CTGGCCTGCGTCCTCAT CTTCACCATCGTG-3′), position 88–117; P2MEL1A (5′-GCCCAGG TTCCACCCGTTGTTAAATATCG-3′), position 260–288; P3MEL1A (5′-GCCATTATATACGGGCTACTGAACCAA-3′), position 874–900; P4MEL1A (5′-TGCGCAGCGTGTCCATCTCACC-3′), position 1074–1093. The primer pairs shown below the diagram are specific for the human Mel1b receptor and consist of P1MEL1B (5′-CGCTGTC CGCGGTGCTCATCGTCACCACC-3′), position 125–153; P2MEL1B (5′-CCAGGGCCCAGCCGTCATAGAAGATG-3′), position 300–325; P3MEL1B (5′AGCCATTTCTTGGGGGTTGATGGC-3′), position 822–846; P4MEL1B (5′-GCCATTGTCTATGGGCTCTTGAACCAA-3′), position 913–939; P5MEL1B (5′-TGCAGCAGACTCTCTCCCACCA-3′), position 1132–1153. The size of the PCR product obtained with each primer pair is indicated. The splice site between exon 1 and 2 of melatonin receptor genes is indicated by the vertical line between transmembrane domains I and II in the diagram. b, bases.

To ensure that solutions used for RT were free of contaminating sequences control reactions were processed without the addition of mRNA. Controls consisting of the PCR mixture only were also included for each PCR performed to check that the PCR components and primers did not contain contaminating sequences.

The PCR products were electrophoresed through 1% agarose gels and Southern blotted onto GeneScreen nylon filters (NEN Dupont Ltd, Stevenage, Herts, UK) using a pressure blotter (Stratagene, La Jolla, CA, USA). A Mel1a oligo probe, designed to hybridize to a site internal to the Mel1a specific PCR primers, was end-labeled with ^{32}P dCTP (Amersham International) by standard procedures utilizing terminal deoxynucleotidyl transferase. The filters were stripped and reprobed with a Mel1b specific oligo probe internal to the Mel1b specific PCR primers. Hybridization was performed at 65 °C in QuikHyb hybridization solution (Stratagene) according to the manufacturer's instructions. The filters were washed to a final stringency of 1 × SSC at 60 °C. PCR products giving a positive hybridization signal were sub-cloned using a pGEM-T vector system (Promega, Southampton, Hants, UK) and DNA was prepared for sequencing to confirm the identity of the PCR products.

In situ *hybridization*

Cryostat sections (10 µm thick) of fetal kidney (20 weeks gestation) were thaw-mounted onto poly-lysine coated

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slides and stored at −70 °C until use. Sections were then fixed in 4% paraformaldehyde (pH 7·4) in 0·1 M PBS treated with 0·1% diethylpyrocarbonate (PBS–DEPC) for 20 min then rinsed twice with PBS–DEPC followed by acetylation with 0·1 M triethanolamine and 0·0025% acetic anhydride for 10 min. After washing twice in PBS–DEPC, sections were dehydrated in a graded series of ethanol–DEPC water solutions before being dried under vacuum for 60 min prior to hybridization with ^{35}S-labeled riboprobes. ^{35}S-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription using the appropriate RNA polymerase in the presence of ^{35}S-alpha-thio-UTP (NEN Dupont Ltd; 1000 Ci/mmol). Templates consisted of fragments at the 3′ end of the human Mel1a and Mel1b melatonin receptor genes generated by PCR of human genomic DNA using the primer pairs P3MEL1A and P4MEL1A and P4MEL1B and P5MEL1B (see Fig. 1). The PCR products were sub-cloned into pGEM-T and the sequences of the riboprobe templates were verified by DNA sequencing. The divergent C-terminal regions were selected to eliminate cross hybridization of probes with the two mRNA species, Mel1a and Mel1b, which show extensive homology over the seven transmembrane domain spanning regions. The C-terminal regions were chosen in preference to the N-terminal regions which contain GC-rich sequence. The tissue sections were overlaid with 70 µl hybridization buffer containing 7×10^3 c.p.m./µl of either Mel1a or Mel1b specific ^{35}S-labeled antisense riboprobes. Adjacent sections were hybridized with the appropriate sense Mel1a or Mel1b specific riboprobe to determine levels of background hybridization of ^{35}S-labeled probe to fetal kidney tissue. The hybridization buffer consisted of 50% formamide, 300 mM sodium chloride, 1 × Denhardt's solution, 10 mM Tris (pH 8·0), 1 mM EDTA, 10% dextran sulphate, 10 mM DTT and 0·5 mg/ml tRNA. Sections were incubated at 55 °C for 16–18 h before being washed four times in 4 × SSC then treated with an RNase A solution consisting of 20 µg/ml RNase A, 500 mM sodium chloride, 10 mM Tris (pH 8·0) and 1 mM EDTA for 30 min at 37 °C. Sections were then washed to a final stringency of 0·1 × SSC at 55 °C followed by dehydration in a graded series of ethanol and then air dried and apposed to Hyperfilm-βMax film (Amersham International) for 2–4 weeks. Sections were stained in toluidine blue to facilitate identification of labeled areas.

DNA sequencing

DNA was prepared for sequencing using a Wizard 373A DNA purification kit (Promega) according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) using universal M13 primers and synthetic oligo primers and an Applied Biosystems model 373 DNA sequencer.

Chemicals and enzymes

All chemical reagents were obtained from Sigma International unless otherwise stated. Restriction and DNA modifying enzymes and enzyme buffers were obtained from Promega or GibcoBRL Life Technologies Ltd, Paisley, Strathclyde, UK. Random and synthetic oligo primers were purchased from GibcoBRL Life Technologies Ltd.

Results

Localization of 2-[^{125}I]iodomelatonin binding in the human fetal kidney

Specific 2-[^{125}I]iodomelatonin binding was found over the nephrogenic region of the outer perimeter of the developing fetal kidney cortex (Fig. 2). A low level of non-specific binding was observed elsewhere in the fetal kidney tissue. Emulsion autoradiographs revealed silver grains over the structures formed during early morphogenesis of the nephron, the comma stage and S-shaped bodies, together with the developing nephrogenic tubules (Fig. 3). Incubation of human fetal kidney sections with 2-[^{125}I]iodomelatonin for increasing lengths of time revealed that specific binding reached equilibrium after approximately 1 h and remained stable thereafter for up to 4 h (Fig. 4). Specific binding increased with increasing concentrations of 2-[^{125}I]iodomelatonin and saturated at around 100 pM 2-[^{125}I]iodomelatonin (Fig. 4). Saturation isotherms were determined for kidneys at 16·5 to 20 weeks. A typical saturation isotherm at 20 weeks gestation is shown (Fig. 4). A one-site binding model revealed a dissociation constant (K_d) of 47 pM and a theoretical maximal binding (B_{max}) of 3 fmol/mg protein. Incubation of sections with increasing concentrations of GTPγS or ATP and 2-[^{125}I]iodomelatonin revealed inhibition of 2-[^{125}I]iodomelatonin binding by GTPγS but not by ATP (Fig. 4).

Expression of melatonin receptors in the human fetal kidney

RT–PCR of the human fetal kidney mRNA with Mel1a and Mel1b specific primers gave PCR products of the appropriate size. Southern blots of the PCR products showed hybridization to the appropriate Mel1a (Fig. 5A) and Mel1b (Fig. 5B) specific oligo probes internal to the PCR primers. DNase treatment of fetal kidney mRNAs prior to RT–PCR did not eliminate amplification of PCR products hybridizing to Mel1a and Mel1b specific oligo probes. No hybridizing PCR products were observed in reagent controls and RT–PCR products were eliminated in samples treated with RNase prior to RT–PCR (Fig. 5). DNA sequencing confirmed the identification of Mel1a and Mel1b melatonin receptor cDNAs from the human fetal kidney RT–PCR products.

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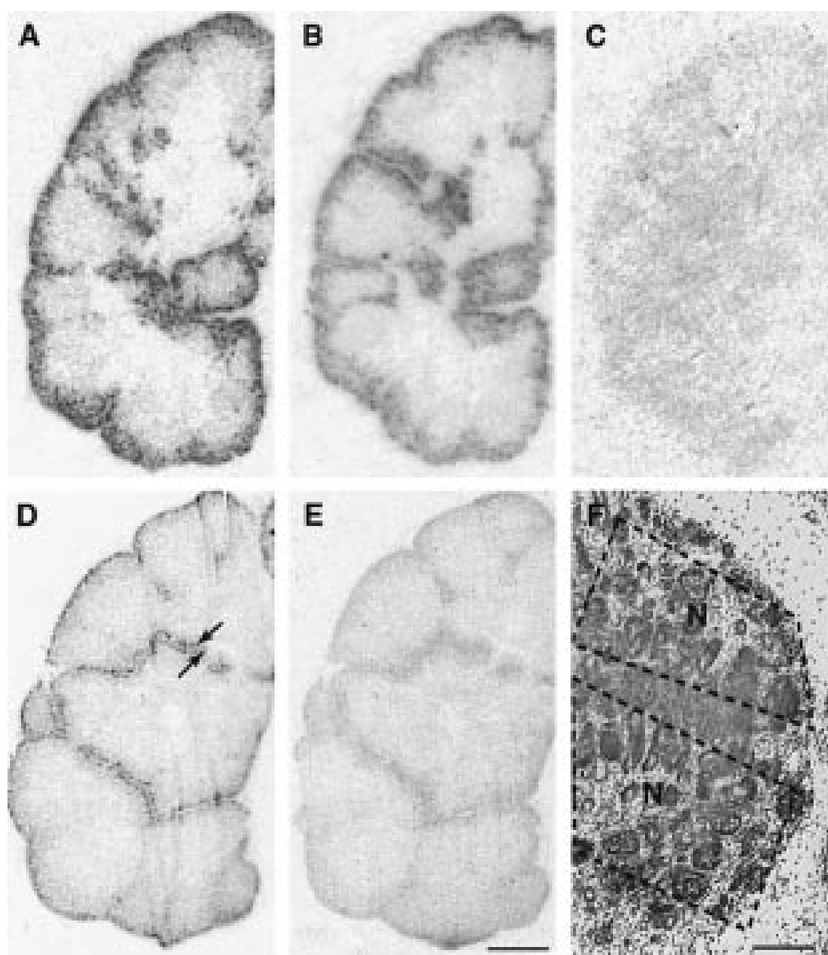

Figure 2 Localization of 2-[^{125}I]iodomelatonin binding and expression of melatonin receptor mRNA in the nephrogenic region of the human fetal kidney (20 weeks gestation). (A) Total binding of 2[^{125}I]iodomelatonin over the nephrogenic region of the human fetal kidney cortex, (B) in the presence of 10^{-4} M GTPγS and (C) in the presence of 10^{-7} M melatonin. Tissue sections showing 2-[^{125}I]iodomelatonin binding were exposed to film for 14 days. (D) [^{35}S] antisense Mel1a specific riboprobe. The arrows indicate localization of Mel1a mRNA expression in the nephrogenic region, see Fig. 2F. (E) [^{35}S] sense Mel1a specific riboprobe. (F) High-magnification of the area indicated by the arrows in Fig. 2D showing the nephrogenic region (N) of the kidney. Tissue sections showing Mel1a mRNA expression were exposed to film for 30 days. Fig. 2A–E, bar=3 mm; Fig. 2F, bar=0·5 mm.

In situ hybridization revealed binding of the antisense Mel1a riboprobe to the nephrogenic region of the outer perimeter of the cortex of the developing human fetal kidney (Fig. 2). The low background of hybridization observed over the developing kidney was similar to that of hybridization to the Mel1a sense riboprobe. *In situ* hybridization using the antisense Mel1b riboprobe failed to localize expression of Mel1b mRNA.

Discussion

We have detected the presence of 2-[^{125}I]iodomelatonin binding sites in the developing human fetal kidney correlated with expression of the mRNA for the G protein-coupled melatonin receptors, Mel1a and Mel1b. Specific 2-[^{125}I]iodomelatonin binding sites were located at the outer periphery of the developing kidney cortex in the nephrogenic region consisting of differentiating and developing nephrons. The binding sites were sensitive to GTPγS suggesting that they represent G protein-coupled melatonin receptors. This was substantiated by the identification of Mel1a and Mel1b mRNA expression by RT–PCR and *in situ* hybridization of the Mel1a melatonin receptor mRNA also localized over the same region. *In situ* hybridization of human fetal kidney with the Mel1b specific riboprobe failed to localize Mel1b mRNA expression. Both the Mel1a and Mel1b specific riboprobes

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Figure 3 Photomicrographs of emulsion-coated slides of human fetal kidney (16·5 weeks gestation) labeled with 2-[^{125}I]iodomelatonin and stained with toluidine blue. (A) Dark field. (B) Light field. Silver grains appear white and can be seen to overlie the nephrogenic region (N) of the kidney. Bar=0·2 mm. (C) High-magnification of section shown in Fig. 3A demonstrating silver grains due to 2-[^{125}I]iodomelatonin labeling over an S-shaped body of a developing nephron (S). Tissue sections were exposed for 60 days. Bar=1·5 µm.

Figure 4 Characterization of 2-[^{125}I]iodomelatonin binding to fetal kidney. Values are derived from quantitative *in vitro* autoradiography. (A) Time-course of specific binding, (B) typical saturation isotherm and (C) the effect of increasing concentrations of GTPγS and ATP on binding.

Figure 5 RT–PCR for Mel1a and Mel1b melatonin receptor gene expression in the human fetal kidney. (A) Lane I shows hybridization of a Mel1a specific radiolabeled oligo probe (5′-TGCGTTCCTGAGCTTCTTGTTCCGATACAC-3′, position 154–183) internal to the PCR primers to a product of the appropriate size using the PCR primers indicated. No hybridizing products were obtained in control lane II, RNase-treated fetal kidney mRNA prior to RT–PCR, lane III, reagents used for RT–PCR with no mRNA added and lane IV, the water control. (B) Lanes I and V show hybridization of a Mel1b specific radiolabeled oligo probe (5′-TGCGTTCCGGAGCTTGCGGTTCCTGAGCAC-3′, position 193–222) internal to the PCR primers to products of the appropriate size using the PCR primers indicated. No hybridizing products were obtained in control lanes II and VI, RNase-treated fetal kidney mRNA prior to RT–PCR, lanes III and VII, reagents used for RT–PCR with no mRNA added and lanes IV and VIII, the water controls. The hybridizing PCR products were sub-cloned and sequenced to confirm identity.

exhibit specific hybridization to transfected cells expressing the appropriate melatonin receptor (data not shown). It may be speculated that levels of Mel1b mRNA expression in the human fetal kidney, detectable by RT–PCR techniques, were too low for detection by *in situ* hybridization. Repeated RT–PCR using Mel1a specific primers revealed products visible on an ethidium bromide stained gel. In contrast the RT–PCR products obtained using Mel1b specific primers were only detected after Southern hybridization. Quantitative RT–PCR analysis will be necessary to determine relative levels of expression of the Mel1a and Mel1b mRNAs.

The developing metanephric (permanent) kidneys undergo a series of complex signaling events which result

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in a gradient of cell differentiation and tissue maturation (for reviews see Davies 1993, Davies & Bard 1996). The induction of development of the excretory nephrons in the cortex of the metanephric kidney is initiated as a result of interactions between the ureteric bud and the metanephrogenic mesenchyme. This interaction causes a mutual induction of the metanephrogenic mesenchyme to develop a stem cell phenotype which differentiates to form nephrons, and bifurcation of the ureteric bud to form the collecting duct system of the kidney (Grobstein 1953, Bard *et al.* 1994). Ultimately the developing kidney cortex consists of a series of cell layers with the innermost cells consisting of maturing nephrons. Towards the outer area of the developing cortex are differentiating and developing nephrons bound by an outer perimeter of undifferentiated stem cells which feed the nephrogenic pathway. The region of developing nephrons is absent in new-born and adult kidneys. The location of 2-[^{125}I]iodomelatonin binding and expression of the Mel1a melatonin receptor in the region consisting of differentiating and developing nephrons is consistent with a role in development of the human fetal kidney.

Specific 2-[^{125}I]iodomelatonin binding in the adult human kidney cortex and in the brush border and basolateral membranes of the proximal tubule in the guinea pig have been reported (Song *et al.* 1995*a*, 1997). Whether these putative melatonin receptors play a role in circadian rhythms of glomerular filtration (Koopman *et al.* 1996) and renin activity (Gordon *et al.* 1966) that have been observed remains to be established. In the fetal kidney it is unlikely that interaction of melatonin with its receptors contributes to renal function as the labeled nephrons are not yet fully developed and functioning in the region exhibiting 2-[^{125}I]iodomelatonin binding and Mel1a melatonin receptor mRNA expression. It may be that the differing pattern of 2-[^{125}I]iodomelatonin binding sites in the human fetal kidney and the adult kidney is indicative of a dual role for melatonin in the kidney.

Acknowledgements

The authors would like to thank Ms A Newman for assistance with DNA sequencing, Dr Julian Mercer for advice regarding *in situ* experiments and Dr Elizabeth Gray (Department of Pathology, Foresterhill Hospital, Aberdeen) for interpretation of the histologic structure. This work was supported by the Scottish Office Agriculture Environment Fisheries Department and a grant from Servier, France.

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Received 11 March 1997
Revised manuscript received 4 August 1997
Accepted 1 September 1997

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