Differential gene expression after implantation of biomaterials into rat gastrointestine

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Two biodegradable materials, polyglycolide-copoly(L)-lactide/poly-dioxanone composite and poly(3-hydroxybutyrate), have been employed for surgery in the gastrointestine of laboratory rats. The tissue response was analyzed at distinct time intervals up to 2 months by differential mRNA display. Two specific PCR fragments were transiently present 7 and 14 days after contact with poly(3-hydroxybutyrate). Both fragments represent distinct rat lipases which might play a role in poly(3-hydroxybutyrate) degradation.

1. Introduction

The utilization of biomaterials in gastrointestinal surgery depends on the biocompatibility of the material and subsequent degradation by the host. Growth factors and/or cytokines are involved in the wound healing process [1, 2] and are expected to be present in the healing process after the biomaterial has been implanted [3]. However, virtually nothing is known about a specific tissue response towards a given biomaterial and whether a specific reaction of the host will lead to rejection, encapsulation or degradation of the biomaterial. After learning about the tissue response to biomaterials and their function in biocompatibility we might be able to either redesign the material or influence the tissue response through medication towards a desirable higher compatibility.

2. Materials and methods

2.1. Surgical procedure

The stomach of Wistar rats was slit and the wound was closed by surgical suture with a polypropylene thread (Resolon®). A patch of poly(3-hydroxybutyrate) (PHB), or polyglycolide-copoly(L)-lactide/poly-dioxanone composite (Vicryl®) was sutured onto the closed wound. After defined time intervals the stomach was removed and tissue prepared for microscopy or frozen in liquid nitrogen and stored at $-80\,^{\circ}C$.

2.2. RNA extraction and mRNA differential display

Frozen tissue samples were transferred to liquid nitrogen and ground with pestle and mortar to a fine powder. The powder was added to extraction buffer/phenol (0.8% triisopropylnaphthalene sulfonic acid, 4,8% *p*-aminosalicylic acid, 0.25 M NaCl, 50 mM ethylene diaminetetraacetic acid (EDTA), 50 mM β-mercaptoethanol, 0.25 M Tris–HCl, pH 9/phenol, 1/1), vortexed and centrifuged [4]. The aqueous phase was re-extracted and nucleic acid precipitated with 70% ethanol. RNAs of four individual tissue samples were pooled and subjected to DNAseI digestion. The RNA amount was determined through OD260 readings.

For the differential display procedure [5,6] 1 μg of RNA was reverse transcribed with Superscript II (Gibco BRL) and anchor primers $T_{12}VA$, $T_{12}VC$, $T_{12}VG$, or $T_{12}VT$ at 2.5 μM (V is either desoxynucleotide A, C or G; MWG Biotech, München) in the presence of 20 μM desoxynucleotide triphosphate (dNTPs) (PeqLab, Erlangen). Polymerase chain reactions (PCR) were performed in the presence of one arbitrary 10-mer primer (0.5 μM, Appligene Oncor, Heidelberg) and anchor primer as above at reduced dNTP concentration of 2 μM and 0.5 μM [^{35}S]dATP (> 3.7 TBq mmol^{-1}, Hartmann Analytik, Braunschweig). A total of 20 arbitrary 10-mer primers were combined with four groups of anchor primers. After initial denaturation of the DNA at 94 °C for 2 min 40 cycles of 30 s at 94 °C, 2 min at 40 °C, 30 s at 72 °C were followed by a final extension step for 5 min at 72 °C in a PTC100 thermal cycler (MJ Research, Watertown, MA, USA). PCR products were electrophoresed through a 6% acrylamide gel under denaturing conditions [7] at 60 W. After electrophoresis the gel was mounted onto GB002 blotting paper (Schleicher & Schuell, Dassel) and dried under vacuum. The dry gel was exposed to X-ray Film (Kodak Biomax) over night.

2.3. Isolation of fragments and DNA sequencing

PCR fragments of interest were cut from the dried gel through the developed X-ray film, rehydrated, boiled for

15 min and then precipitated with 70% ethanol in the presence of 0.17% glycogen [6] at −20 °C. DNA was pelleted by centrifugation, washed with 70% ethanol and finally redissolved in 10 μl H_2O. Four microliters of the DNA was amplified in a PCR under the same condidions employed for the differential display procedure. Then, an aliquot was removed and amplified in another 40 cycles of PCR and the DNA electrophoresed in a 1% agarose gel. The band of interest was cut from the gel and the DNA was isolated with NuceloSpin Extract (Macherey & Nagel, Düren). DNA fragments were sequenced directly by cycle sequencing primed with the appropriate 10-mer primer in the presence of fluorescently labeled dideoxy-nucleosidtriphosphates (ABI Prism, PE Applied Biosystems). After initial denaturation for 2 min at 96 °C 25 temperature cycles of 2 min at 94 °C, 4 min at 40 °C, and 4 min at 60 °C were run in a Mastercycler Gradient (Eppendorf, Hamburg). Unincorporated nucleotides were removed with Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA). DNA fragments were then sequenced in an ABI Prism 310 Genetic Analyser (PE Applied Biosystems).

3. Results

After macroscopic and microscopic evaluation of the tissue response towards PHB we were interested to learn about the tissue response at the cellular and molecular level. The pattern of active genes is reflected by the presence of the individual mRNAs. RNAs isolated from the tissue were intact, as judged by agarose gel electrophoresis showing two clear bands of the 18S and 28S rRNA (data not shown). Reverse transcription and amplification of subsets of cDNAs by PCR produced patterns of PCR fragment lengths. The majority of the fragments were obtained from all RNAs irrespective of former tissue treatment (Fig. 1). Two PCR fragments (#27 and #61) were visible in samples obtained from tissue that has been in contact with PHB for 7 and 14 days but the same fragment was absent under all other experimental conditions (Fig. 1). Each fragment was sequenced, and in both cases homology to rat lipase cDNAs was found. Fragment #27 shares 99% sequence identity with a rat lipase cDNA and fragment #61 shares 85% sequence identity with a rat triglyceride lipase.

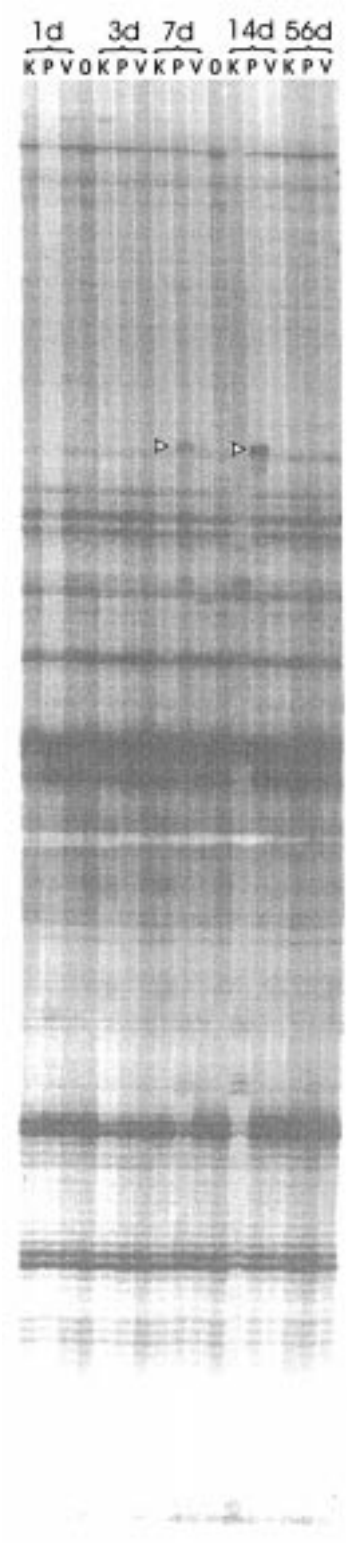

Figure 1 Differential display. Autoradiograph of an acrylamide sequencing gel displaying PCR fragments. Lanes labeled with 0 contain PCR fragments representing mRNA isolated from healthy rat stomach tissue. Lanes labeled C show PCR fragments representing mRNA from tissue subjected to the surgical procedure alone. Lanes marked P or V show PCR fragments representing mRNA from tissue which has been in contact with PHB or Vicryl, respectively. Times at which the tissue was removed for analysis are indicated in days. The primers used for this experiment were the anchor primer $T_{12}VG$ and the 10-mer primer GCAACGTCGG. A pool of four individual RNA preparations was reverse transcribed and the resulting cDNA served as a template for the PCR reactions. The two signals in lane P at 7 and 14 days (arrowheads) represent an mRNA that is transiently present 7 and 14 days after PHB implantation, but is not displayed in tissue samples obtained from rats implanted with vicryl (lanes V) or subjected to the surgical procedure alone (lanes C) or untreated animals (lanes 0).

4. Discussion

4.1. Differential display

Various patterns of DNA fragments produced in the PCR reactions have been observed. Not all RNA samples have been free of genomic DNA contamination, as judged in selected PCR assays omitting the reverse transcription step (data not shown), such that the rise of amplifying genomic sequences cannot be excluded. However, the two PCR fragments isolated here (#27 and #61) show the $T_{12}GG$ and $T_{12}GT$ sequence at their 3′-end and the cDNA sequences show a perfect match within a stretch of eight nucleotides with the arbitrary 10-mer primers. Experiments are underway using specific primer pairs to confirm by reverse transcription (RT)–PCR the presence of the two mRNAs.

Fragment #27 represents an mRNA for lipase with

99% sequence identity to rat lipase. It is conceivable that the nucleotide mismatch is due to a sequencing error rather than the presence of another allele in Wistar rats. In the case of fragment #61 the limited sequence identity is in part due to unidentified nucleotides in the sequencing reaction and most likely to further sequencing errors due to the use of the 10-mer primer. The presence of other alleles is highly unlikely as both genes are listed as unique rat sequences in Genbank.

4.2. Putative function of lipases in biocompatibility

Questions concerning the function of the transiently expressed lipases in the context of the tissue response towards biomaterials cannot be answered unambiguosly at this time. Most intriguing is the observation that the active site of lipases resembles the active site of PHB depolymerases [8] and therefore one might argue that lipases are involved in biodegradation of PHB.

4.3. Specificity of the tissue response

The fact that the mRNAs appear transiently 7 days after implantation of PHB might reflect that other cellular responses are needed within hours of implantation, finally leading to expression of the lipases. In addition, special cues might be necessary to downregulate the mRNA amount 14 days after implantation. In all scenarios there is a requirement for a specificity which implies that the cells in contact with the PHB can identify PHB. Whether there are specific receptors for PHB or whether other receptors are recruited for the recognition of PHB remains to be determined. The fact that there is a specific response towards PHB might open ways to block or stimulate the tissue response using this signalling pathway in order to improve biocompatibility or tailor the speed of the biodegradative process.

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