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Antisense down regulation of connexin31.1 reduces apoptosis and increases thickness of human and animal corneal epithelia

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Abstract

The roles of the gap junction protein connexin31.1 (Cx31.1) are poorly understood, especially as the protein appears to form non-functional channels. Cx31.1 specific antisense oligodeoxynucleotides (ODNs) were designed to evaluate its roles in a corneal epithelium model. Expression of Cx31.1 in corneal epithelium extends from the suprabasal layers of polyhedral wing cells through to the flat squamous cells of superficial layers which are shed into the tear film. Deoxyribozymes (Dzs) were tested for cleavage efficacy using *in vitro* transcribed Cx31.1 mRNA. Cleavage results showed a putative tertiary structure for Cx31.1 mRNA with one region appearing to have a higher potential for antisense targeting. Application of antisense ODNs designed to this region caused Cx31.1 knockdown in rat and human corneal organotypic culture models, leading to a reduction in apoptosis and a thickening of the corneal epithelium ($p = 0.0045$). Cx31.1 appears to play a role in triggering cell death; knocking it down may provide a novel approach for tissue repair and engineering.

Keywords: Connexin; Apoptosis; Epithelium; Cornea; Antisense oligodeoxynucleotides; Cx31.1; Gap junction

1. Introduction

Gap junction channels connect the cytoplasm of a cell directly with that of adjoining cells. This intercellular communication permits coordinated cellular activity, such as the sharing of ions, secondary messengers, and small metabolites. The exchanged ions and small molecules must be less than approximately 1 kDa (Kelsell et al., 2001; Kumar and Gilula, 1996) in mass in order to pass through the channels without involving the extracellular space. Intercellular channels are formed from two integral membrane hemichannels, one from each of the adjacent cells, called connexons. Each connexon is formed by six connexin protein subunits. Connexons may be either homotypic or heterotypic (Goodenough et al., 1996; Simon and Goodenough, 1998). There are at least 20 connexin isomers in the human, forming a highly regulated multigene family (Kumar and Gilula, 1996). Despite the large number of studies completed on members of the connexin family, there is relatively little work focusing on connexin31.1 (Cx31.1). Cx31.1 is a relatively rare gap junction protein, and appears to be unique in its inability to form functional gap junction channels, either with itself or with other connexin isoforms (Gilula et al., 1999; Harris, 2001). It is expressed in the middle and outer layers of the corneal epithelium (Laux-Fenton et al., 2003) and skin epidermis (Goliger and Paul, 1994; Hennemann et al., 1992), with minor levels expressed in testis (Hennemann et al., 1992). Cx31.1 is believed to have a dynamic expression pattern in rat epidermis throughout developmental stages (Goliger and Paul, 1994) and after wounding (Coutinho et al., 2003; Goliger and Paul, 1995). It is closely linked to Cx30.3 on mouse chromosome 4 with Cx30.3 mutations shown to cause the skin disease erythrokeratoderma variabilis (EKV) (Macari et al., 2000). While EKV might also involve mutations in Cx31.1, no evidence for this has been found. Cx31.1 is known to be strongly inhibited during all stages of mouse skin carcinogenesis (Budunova et al., 1995), but conversely, expressed in

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Abbreviations

Cx	connexin
DMEM	Dulbecco's modified Eagle's medium
Dz(s)	deoxyribozyme(s)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EKV	erythrokeratodermia variabilis
kDa	kilodalton
ODN(s)	oligodeoxynucleotide(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBE	Tris/Borate/EDTA

atretic ovary granulosa cells which are about to undergo apoptosis (Wright et al., 2001).

Development of antisense oligodeoxynucleotides (ODNs) as pharmacological tools and as therapeutic agents (Crooke and Bennett, 1996; Lebedeva and Stein, 2001; Wagner, 1994) has grown rapidly in the last decade. Antisense ODNs provide a potentially powerful and highly specific molecular technique to knockdown selected protein expression. We have shown previously that treatment of skin wounds with Cx43 antisense ODNs improved wound closure and lowered the inflammatory response compared to control wounds (Qiu et al., 2003). A similar response and improved healing results in corneas after excimer laser keratectomy have also been observed following Cx43 specific antisense ODN treatment (Laux-Fenton, 2003).

We have developed Cx31.1 specific antisense ODNs to assess the biological function of Cx31.1 using a corneal epithelium model. The transverse stratification of corneal epithelial cells is strikingly similar to the differentiation processes that occur in the epidermis of the skin and both share a common embryonic origin (Gilbert, 1988a,b). Corneal epithelium has 6–8 cell layers yet expresses different connexins in different layers, with Cx31.1 in the upper suprabasal and more superficial cell layers where cells are destined to die and be shed (Laux-Fenton et al., 2003). We show that Cx31.1 specific antisense ODNs reduced Cx31.1 expression in *ex vivo* corneal organ cultures, leading to cell retention within the zone between the suprabasal cells and the surface. The effect is a decrease in the number of epithelial cells undergoing apoptosis and an increase in the number of epithelial cell layers, causing a significant thickening of the corneal epithelium.

2. Materials and methods

2.1. Tissue preparation

Approval for animal work was obtained from the University of Auckland Animal Ethics Committee and for human tissue-based research approval was obtained from the Northern X Regional Human Ethics Committee. A total of 80 eyes (40 matched pairs) of P30 Wistar rats and 1 matched pair of human corneas were used for this study. Rat eyes were enucleated, rinsed in PBS (OXOID Ltd, Hampshire, England) and embedded in Tissue-Tek® OCT (Sakura Finetek, Torrance, CA, USA) before being snap frozen in liquid nitrogen. Corneal cryosections (16–25 μm) were cut orthogonally to the corneal surface using a Leica cryostat and mounted on electrostatic slides (SuperFrost® Plus, Menzel–Glaser, Germany). Fresh normal human corneal tissue was obtained from the New Zealand National Eye Bank (Auckland, NZ) using corneas deemed unsuitable for transplantation. Corneas were fixed in 4% fresh paraformaldehyde for 1 h at 4 °C or snap frozen in liquid nitrogen after cutting the corneas in half. Corneal cryosections were cut 10–16 μm in thickness and were immediately mounted on electrostatic slides. For structural analysis, frozen sections were stained using Mayer's haematoxylin and eosin (1%) following standard histological procedures and mounted in DEPEX (GURR, Germany).

2.2. Immunohistochemistry

Corneal cryosections were washed in PBS then incubated with rabbit anti-Cx31.1 primary antibodies at 1:1000 dilution or cell proliferation monoclonal marker Ki67 (1:100; Immunotech, Marseille, France) overnight at 4 °C. Cx31.1 antibodies were raised to a synthetic 15 residue peptide matching amino acids H–HPPLLPDRPRAHVKK–OH of the rat Cx31.1 protein sequence by Research Genetics Inc. (USA). Sections were rinsed in PBS and incubated with a 1:20 dilution of Swine anti-rabbit FITC secondary antibody (Dako, Denmark), a goat anti-rabbit IgG Alexa Flour® 568 secondary antibody (Molecular Probes, OR, USA) at 1:200 dilution, or a goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, INC., West Grove, PA, USA) at 1:400 for 2 h at room temperature. Tissues were then washed in PBS before mounting in Citiflour™ (Agar Scientific, UK). The fluorescence labelling was observed with Leica SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

2.3. Deoxyribozyme design

Oligodeoxyribozymes (Dzs) and ODNs were designed using similar methods described in previous studies (Cairns et al., 1999; Santoro and Joyce, 1997). All AT and GT sites after the start codon in the sense cDNA sequence of Cx31.1 were selected with 9 nucleotides left either side of the A or G. The antisense Dzs are the complement of the sense sequence with the "A" or "G" replaced with the autocatalytic core "ggctagctacaacga". Three essential requirements for Dzs design are:

2.3.1. Thermostability

Any Dz with a melting temperature above 30 °C was ignored, because over this temperature 50% of the antisense Dzs are likely to form stable secondary hairpin configurations