

# In situ immunohistochemical study of Bcl-2 and heat shock proteins in human corneal endothelial cells during corneal storage

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## Abstract

**Aim**—To investigate the expression of Bcl-2 and heat shock proteins (HSPs), which are known to increase cell survival, in human corneal endothelial cells (HCECs) of corneas stored in organ culture.

**Methods**—32 paired corneas were randomly assigned to either a short or a long storage time. The flat mounts of endothelium were examined after immunostaining with monoclonal antibodies to Bcl-2 and HSP 27, 60, 70, and 90.

**Results**—HCECs expressed generally all the proteins studied. Bcl-2 expression was weaker in the long stored corneas ( $p=0.035$ ). There was no relation between immunostaining, age, sex, or death to culture time. Frequently some Descemet membranes carried negative cells preferentially located in folds and exhibiting morphological changes consistent with swelling cells corresponding to early stages of apoptosis.

**Conclusion**—Expression of these cytoprotective proteins reflects the high level of HCEC resistance to stresses induced by organ culture. The decreased immunostaining of Bcl-2 in the long storage group could act in cellular loss currently observed with storage time. The negativity of Bcl-2 and HSP labelling in corneal folding may be related to apoptosis.

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increase of intraocular pressure, genetic endothelial dystrophies, trauma, and surgical stresses. Corneal graft is a situation in which a combination of almost all of these factors is encountered during the different steps from donor death and corneal conservation, to the graft and postoperative immunological host-graft reactions, which determine HCEC loss. It has already been demonstrated that HCEC death mechanisms may involve necrosis and/or apoptosis in circumstances including corneal storage.<sup>3 4</sup>

Cell death is controlled by proteins such as Bcl-2 and heat shock proteins (HSPs) which suppress either necrosis or apoptosis.<sup>5</sup> The oncogene *bcl-2* was first identified at the chromosomal breakpoint of t(14;18) bearing B cell lymphomas.<sup>6</sup> The Bcl-2 protein extends cell survival rather than stimulating cell proliferation.<sup>7</sup> Bcl-2 has been shown to enhance cell survival by inhibiting apoptosis induced by a wide range of stresses: ultraviolet light exposure, heat shock, reactive oxygen species, viral agents, various drugs, and growth factor deprivation. This suggests that Bcl-2 is a ubiquitous inhibitor of cell death triggered by multiple routes.<sup>8 9</sup> Bcl-2 may act in more than one way to prevent apoptosis depending on cell type, apoptosis inducers, and Bcl-2 cellular localisation: Bcl-2/Bax (Bax is a proapoptotic homologue of Bcl-2) bindings prevent formation of proapoptotics homodimer Bax/Bax.<sup>10</sup> Bcl-2 is an antioxidant,<sup>11</sup> and Bcl-2 prevents the mitochondrial outcome of proapoptotic molecules like cytochrome C or apoptosis inducer factor.<sup>12</sup> In the cornea, the protein Bcl-2 is thought to play a major part in protecting against mechanisms resulting in HCEC death. It is demonstrated that the Bcl-2 messenger RNA is present in HCECs<sup>13</sup> and that the expression of the protein Bcl-2 in HCECs is upregulated by a not yet identified protein aqueous humour factor.<sup>14</sup>

Cell protection against stresses involves other proteins, especially heat shock proteins (HSPs). These are classified in two categories according to their molecular weight: major HSPs (HSP60, 70, 90) and small HSPs including HSP27. HSPs have a highly conserved structure and are constitutively expressed in most cells under physiological conditions having a role in cell homeostasis. HSP27 and HSP70 are characterised by large domains of homology to  $\alpha$ A, $\beta$ -crystallin protein. This is the major structural protein of vertebrate eye lens and is also present in almost all

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Corneal transparency is largely dependent on the integrity of the endothelial monolayer. Human corneal endothelial cells (HCECs) share with other cells, like neurons, a very long lifespan. Physiological decrease in cell density is 0.6% a year.<sup>1</sup> In return, their capacity for self renewal is insignificant and wound healing is thus achieved by migration and enlargement of residual cells in order to maintain monolayer integrity and sufficient deswelling capacity.<sup>2</sup> In contact with aqueous humour, HCECs are exposed to cytotoxic stresses such as free oxygen species induced by ultraviolet radiation, physical trauma, and temperature variation. In normal conditions, cellular death is likely to be strictly repressed in order to prevent extensive cell loss and conserve corneal transparency. Nevertheless, multiple pathological conditions are likely to be responsible for dramatic cell loss, such as inflammation,

other tissue having a chaperone role in cell protection mechanisms.<sup>15 16</sup> Exposure to multiple cellular stresses, like sublethal heat shock, anoxia, and exposure to cytokines, induces HSPs expression which protect cells from either necrosis or apoptosis in many cultured cells and animal tissues.<sup>5 17 18</sup> Cell protection is mediated by their capacity to function as molecular chaperones: HSPs prevent inappropriate protein aggregation, accelerate recovery from heat induced RNA and protein shut-off or nuclear protein aggregation, and mediate transport of immature proteins to the target organelles for final packaging, degradation, or repair.<sup>19</sup> Interestingly, the interaction of HSPs with apoptotic process is also involved in cell protection and was largely investigated.<sup>5 20-30</sup> In the case of protection against apoptosis, HSP27 and other small HSPs may act in regulating the glutathione level in stressed cells by being a specialised chaperone for enzymes involved in the radical oxygen species-glutathione pathway. It is likely that small HSPs act in concert with major HSPs since HSP70 has been reported to protect against different types of stress induced apoptosis.<sup>18 31</sup> HSP27 and Bcl-2 both modulate radical oxygen species and glutathione levels and can thus act at complementary levels in cell protection and apoptosis inhibition.<sup>5 11</sup> Links between Bcl-2 and HSP70 have also been raised recently, suggesting potential implication in the regulation of programmed cell death.<sup>32</sup>

HCECs are well protected against cell death *in vivo* but present a high death rate during corneal storage, particularly after extended time.<sup>33</sup> We hypothesised that conservation conditions, especially after long storage, could influence the expression of protective proteins. The aim of our work was to study the expression by HCEC of proteins involved at different levels in cell protection: Bcl-2, a small HSP (HSP27) and three major HSPs (HSP60, 70, and 90) in human corneas after different storage times in organ culture, which is the most common technique used for corneal storage in European countries. To our knowledge, the expression of HSPs and their links with potential protective effects during organ culture of human corneas has not been investigated.

## Materials and methods

### HUMAN CORNEAS

Human corneas from seven males and nine females aged 29–90 (mean 76 (SD 15) years) were obtained from the Saint-Etienne Eye Bank. Each cornea had an endothelial cell density higher than 2000 cells/mm<sup>2</sup> but was unsuitable for transplantation because of medical contraindications. Mean time between death and corneal harvesting was 18 (SD 7) hours (range 3–34 hours). After *in situ* corneal harvesting, corneal buttons were immediately placed in a 100 ml bottle of Inosol (Chauvin-Opsia, Toulouse, France), organ culture medium containing 2% of fetal calf serum, and stored at 31°C. Paired corneas were randomly assigned to a short storage group (from 6 to 11 days) or a long storage

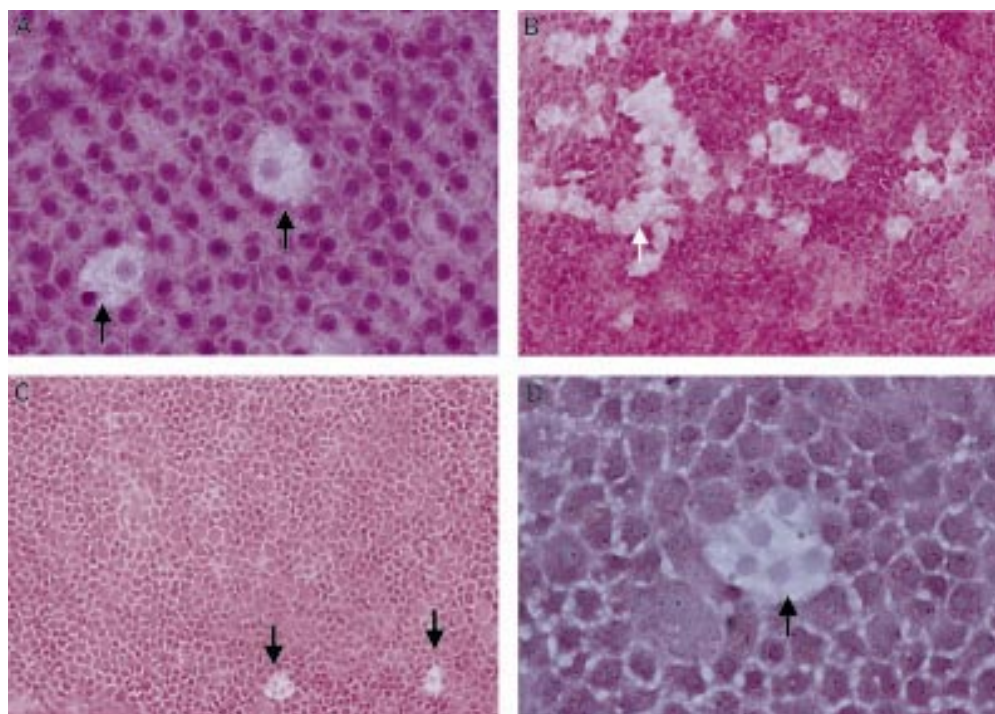
group (from 30 to 35 days). Organ culture medium was renewed at day 2 for all conserved corneas and day 14 for the long storage group. The study was conducted according to the tenets of the Declaration of Helsinki.

### ENDOTHELIUM HARVESTING

Flat mounts of endothelial cells were prepared as follows: cornea was first cut in six parts using a surgical blade. After precutting of the peripheral Descemet membrane with a 9 mm diameter trephine, the Descemet membrane with overlying endothelial cells was gently peeled off under the operating microscope using a monofilament forceps. Pieces of Descemet membrane carrying cells were removed from each cornea and, under the operating microscope, placed endothelium up on precoated slides (Superfrost plus, Menzel-Glaser, Germany), then dried for 24 hours at room temperature, fixed in pure acetone at room temperature for 10 minutes and then stored at –20°C until study by immunocytochemistry.

### IMMUNOCYTOCHEMISTRY

Slides were thawed at room temperature and rehydrated for 5 minutes in phosphate buffered saline (PBS). Slides were incubated for 45 minutes at room temperature with monoclonal mouse anti-human antibody against Bcl-2 (1/40 dilution; Dako, Glostrup, Denmark), HSP27 (1/50 dilution; Santa-Cruz Biotechnology, Santa Cruz, CA, USA), HSP60 (1/100 dilution; Neomarker, Union City, CA, USA), HSP70 (1/100 dilution; Santa Cruz), HSP90 (1/50 dilution; Transduction Laboratories, Lexington, KY, USA). Revelation employed an ABC immunoalkaline phosphatase staining (Vectastain, Vector Laboratories, Burlingame, CA, USA). Slides were incubated 20 minutes with blocking horse serum, then incubated with biotinylated horse antimouse secondary antibody for 30 minutes, rinsed for 5 minutes in PBS, incubated for 30 minutes with streptavidin-biotinylated alkaline phosphatase complex, and developed to yield a red granular reaction product with a substrate solution containing Vector red (Vector Laboratories). The nuclei of the endothelial cells were counterstained with Meyer's haematoxylin (Dako) for 1 minute, rinsed in PBS, and mounted in Fluorotech aqueous media (Valbiotech, Paris, France). Controls consisted of replacement of primary monoclonal antibody by an irrelevant antibody of the same isotype or by PBS. Slides were examined using  $\times 10$  magnification by two observers (PG and GT) in masked fashion regarding donor data and storage time. Cells were considered stained if any diffuse reddish cytoplasmic staining could be identified. A scale of three levels of red staining was used to assess intensity of staining: 0 for no staining, 1 for weak staining, 2 for strong staining. For a technical reason (Descemet detachment from the slide), immunocytochemistry was not possible in one case for Bcl-2 and HSP27, three cases for HSP60, and five cases for HSP90.



**Figure 1** Immunocytochemical staining of HCECs. The staining pattern for Bcl-2 and HSPs was mostly diffuse but displayed frequently some negative cells either isolated (black arrows) or in corneal fold (white arrow) whatever the protective protein labelled. Area of negative cells (A, D) exhibited morphological modifications consisting of increased size of cells, increased size of nucleus, and disappearance of nucleolus. A = Bcl-2, B = HSP27, C and D = HSP70. Original magnification  $\times 10$  (B, C) and  $\times 40$  (A, D). Nuclei were counterstained by Meyer's haematoxylin.

#### STATISTICAL ANALYSIS

The expression of Bcl-2 and HSPs between the randomised groups of short and long storage time was compared using the Wilcoxon matched pairs signed ranks test. The relations between the staining of Bcl-2, HSPs, donor sex, age, and time between death and corneal harvesting were studied on the overall series of corneas using non-parametric tests (Spearman test, Mann-Whitney test). The null hypothesis was tested using two tailed tests. Statistical analysis was performed using the Statistical Package for the Social Science program (SPSS), with p value below 0.05 being regarded as significant.

#### Results

HCECs generally expressed all the proteins studied (Fig 1): considering all the corneas, Bcl-2 was expressed in 77% of them, HSP27 in 97%, HSP60 in 59%, HSP70 in 88%, HSP 90 in 85%. Strong staining was frequently observed for HSP27 (25/31, 81%) and HSP70 (18/32, 56%). All staining was confined to the cytoplasm. Control slides did not exhibit immunostaining. For all corneas, without considering storage time, no significant correlation was found between sex, time between death and corneal harvesting, and intensity of staining. The immunolabelling pattern was not significantly influenced by age.

Following random assignation of the corneas to two groups of storage time (long/short) (Fig 2), intensity of the staining was weaker for Bcl-2 ( $p=0.035$ ) in the long storage group, whereas there was no significant difference for HSPs.

Among the 16 pairs of corneas, the overall expression of HSPs tended to decrease in seven pairs (all staining for HSPs being weaker in the long stored cornea than in the short stored one), whereas it tended to increase in five pairs and remained unmodified in four pairs.

Frequently, some Descemet membranes carried negative HCECs for Bcl-2 and HSPs, either isolated or in areas corresponding to folds induced by stromal swelling during storage and observed on the corneas before Descemet membrane harvesting. Folds were easily identified on the whole cornea under the operating microscope and remained visible after Descemet membrane harvesting and flat mounting. These unstained HCECs had an increased size and presented morphological changes in their nucleus: increased size, fine granular chromatin, and disappearance of nucleolus (Fig 1A, D). These cells corresponded to swelling cells. They were still adherent to the Descemet membrane but appeared sometimes not in the same plane as stained cells and seemed about to desquamate.

#### Discussion

Using an immunocytochemical technique on flat preparations of HCECs, we have demonstrated the presence of Bcl-2, HSP27, 60, 70, and 90 in HCECs from stored human corneas and the decrease in Bcl-2 with long storage time.

The aim of organ culture is to ensure both graft security in allowing the performance of microbiological tests and graft quality in preserving viability of corneal cells. The study of Bcl-2 and HSP expression in organ cultured

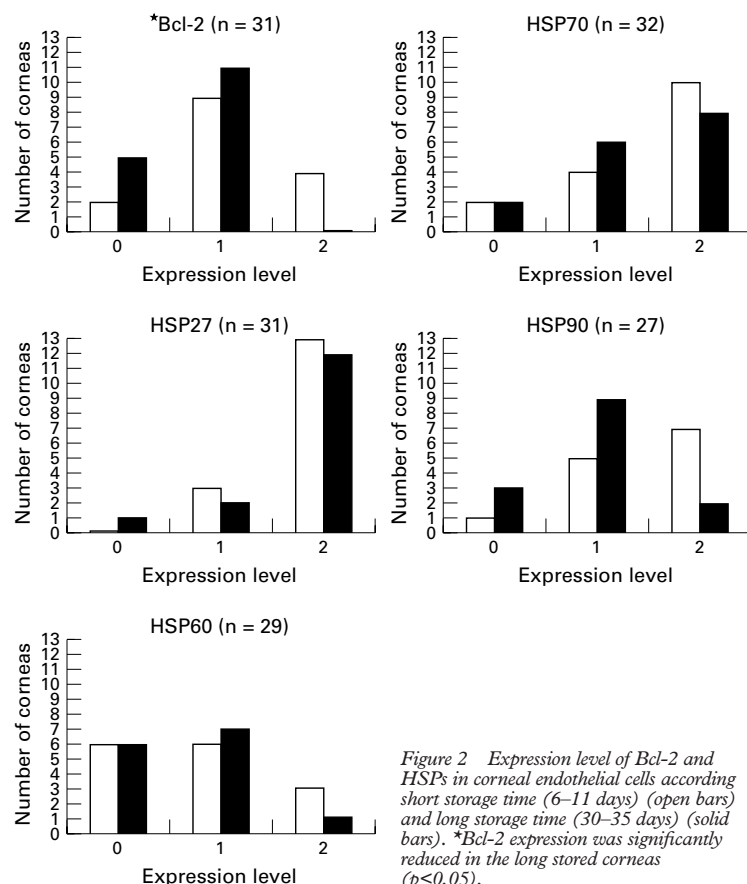


Figure 2 Expression level of Bcl-2 and HSPs in corneal endothelial cells according short storage time (6–11 days) (open bars) and long storage time (30–35 days) (solid bars). \*Bcl-2 expression was significantly reduced in the long stored corneas ( $p < 0.05$ ).

corneas is thus of interest, since they are known to protect against cell death. Bcl-2 messenger RNA was previously located in all cell types of human corneas.<sup>13</sup> Bcl-2 has recently been demonstrated to protect bovine corneal endothelial cells against apoptosis induced by staurosporin (a broad spectrum kinase inhibitor)<sup>34</sup> and its expression in mouse and human corneal endothelium is stimulated by a protein factor present in aqueous humour.<sup>14</sup> We suppose that the lack of this factor in organ culture medium could be an explanation for the decrease in Bcl-2 level in endothelium of long stored corneas in our experiments. This decrease, as a result of a downstimulation of Bcl-2 transcription, renders HCECs more susceptible to apoptosis and could have a role in the usual cell loss observed with extended storage time.<sup>33</sup>

In this study, HCECs have been shown to express different HSPs involved in protection against cell death. Since HCECs are structurally and functionally crucial for corneal function and exhibit non-mitotic characteristics, they may require high constitutive levels of protective proteins. HSP expression is likely to be required in HCECs that must be resistant to cell death despite the numerous environmental stresses encountered in organ culture conditions. Whether this expression, observed during organ culture, is constitutional and/or induced remains unclear. The metabolic, oxidative, heat, mechanical, and immunological stresses present between donor death and the graft could stimulate the expression of

HSPs. Interestingly, in our study, corneas seemed to reveal a heterogeneous response to organ culture, suggesting a heterogeneous cellular response to cell stresses. Some of them had a higher expression of HSPs at the end of storage than at the beginning while others had a lower one, unrelated to donor age or postmortem time. We suppose that corneas do not react in a standard way to the stresses generated by organ culture. This could correspond to the clinical aspect of corneal grafting which also proves heterogeneous *in vivo*, with unequal cell loss rate after grafting independently of recipient parameters.<sup>35, 36</sup> Corneal storage in organ culture could act as a selective test for corneas: corneas that resist cell stresses encountered at all the steps of the storage process, by developing adapted cellular responses like HSP overexpression, could be better, once grafted, than those with less adapted response during storage.

A mosaic pattern of immunostaining was found when the flat HCEC preparations were stained for Bcl-2 or HSPs: among diffuse staining, some cells remained negative, either isolated or located in folds generated by stromal swelling during storage. We suggest that lack of expression for Bcl-2 or HSPs is related to cell death. Unstained HCECs with Bcl-2 and HSP antibodies exhibit morphological changes consistent with swelling, suggesting apoptotic cells. A previous study<sup>37</sup> actually demonstrated that adherent cells present particular morphological features of apoptosis including steps of swelling and rounding before desquamation, and thus do not correspond to gold standard morphological features of apoptosis described in other cell types. In this study the authors used a model of induced apoptosis on adherent cell line HT29 derived from human adenocarcinoma of the colon. Cells exhibit four distinct morphological and biochemical stages of apoptosis: (1) preapoptotic cells, (2) swelling cells detaching from each other but not from the tissue culture plate, (3) rounding cells, and finally (4) floating cells.

Involvement of apoptosis during human corneal storage was recently demonstrated in endothelial cell death at 4°C,<sup>3</sup> 31°C,<sup>38</sup> and 34°C.<sup>4</sup> Furthermore, the location of negative cells for Bcl-2 and HSPs in our study, either in patches or in corneal folds fits with the location of cellular death currently observed during corneal assessment in banks, with vital staining such as trypan blue identifying dead cells either isolated at the centre of rosette formation<sup>39, 40</sup> or in corneal folds.<sup>33, 41, 42</sup> Recent studies using terminal deoxynucleotidyl transferase mediated dUTP nick end labelling technique, which identifies internucleosomal DNA breakdown consistent with apoptosis,<sup>43</sup> found apoptotic cells in the same locations.<sup>4, 38</sup>

By analogy with apoptosis of epithelial adherent cells induced by loss of contact with the basement membrane,<sup>44</sup> stimulus such as loss of contact with the Descemet membrane, induced in corneal folds by stromal swelling encountered after prolonged organ culture, may trigger HCEC apoptosis because of decreasing synthesis of cytoprotectors like

Bcl-2 and/or HSPs. This hypothesis remains to be investigated.

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