Further evidence for heterozygote advantage of GJB2 deafness mutations: a link with cell survival

J E A Common, W-L Di, D Davies, D P Kelsell

METHODS

Full length human wildtype (WT) Cx26 and Cx30 were independently cloned into the pEGFP-N3 plasmid (BD Biosciences Clontech). The disease-associated connexin mutations were introduced into either WT-Cx26 or WT-Cx30 by site-directed mutagenesis (SDM) using the QuickChange SDM kit (Stratagene) according to the manufacturer’s instructions. All positive clones were identified by restriction enzyme analysis and DNA sequenced to check that no erroneous sequence changes had occurred. These constructs were transfected into either NEBI keratinocyte or NIH 3T3 fibroblast cell lines using the transfast reagents according to the manufacturer’s instructions (Promega). After 48 h cells were harvested with the culture medium in order to collect all live and dead cells. Cells were then stored on ice until fluorescence-activated cell scanning (FACS) analysis. Propidium iodide (PI) was added to the cells 2 min prior to analysis. For each sample 10 000 EGFP positive cells were FACS analysed with the percentage of cell death indicated in the key points.

RESULTS AND DISCUSSION

Previously it has been shown that distinct disease-associated mutations within the same connexin have different effects on subcellular protein localisation and on cell viability. Here, we extend our analyses to the effect of connexin mutation on cell survival. Though elevated cell death is associated with skin disease-associated connexin mutations, cells transfected with the deafness-associated GJB2 mutations resulted in reduced keratinocyte cell death compared to the wildtype Cx26 protein (fig 1) and dramatically less than the skin disease-associated GJB2 mutations (fig 1). This observation may hint towards a cellular mechanism supporting a putative selective epidermal advantage for hearing loss-associated Cx26 mutations in different populations. Reduced cell death may extend the keratinocyte terminal differentiation programme resulting in a slightly thicker epidermis. Though, as

Key points

- Mutations in the GJB2 gene that encodes the gap junction-associated protein connexin 26 (Cx26) are the major cause of autosomal recessive non-syndromic hearing loss (NSHL). The high carrier frequency of the GJB2 mutations in many ethnic groups suggests there may be heterozygous advantage.
- A previous study has shown a link with the skin, specifically a thicker epidermis in heterozygotes and homozygotes for the GJB2 mutation R143W.
- To further investigate the functional aspect of heterozygous advantage occurring with GJB2 NSHL mutations we have FACS analysed immortalised keratinocyte cells and NIH 3T3 cells expressing various Cx26-EGFP fusion proteins. Exposure to propidium iodide before FACS analysis allowed cell death status to be quantified.
- We demonstrate that NSHL-associated GJB2 mutations increase cell survival and thus may explain the thicker epidermis due to an extended terminal differentiation programme leading to an improved barrier against infection.

DEAFNESS-ASSOCIATED MISENSE GJB2 MUTATIONS

Cx26 was detected in the NIH 3T3 fibroblast cells by immunocytochemistry, but previous experiments demonstrated a high cell death count in this cell type upon expression of Cx31 skin disease-associated constructs.

Abbreviations: CF, cystic fibrosis; Cx, connexins; FACS, fluorescence-activated cell scanning; NSHL, non-syndromic hearing loss; SDM, site-directed mutagenesis; WT, wildtype

LETTER TO JMG

G ap junctions composed of connexins (Cxs) are intercellular channels that provide a mechanism of synchronised cellular response facilitating the metabolic and electronic functions of the cell. At least 20 human Cx genes have been described, many of which harbour germline mutations that are associated with a variety of human diseases.1 Recessive mutations in the coding region of GJB2 encoding Cx26 are associated with non-syndromic hearing loss (NSHL)2 and, indeed, account for a significant proportion of NSHL worldwide. Within different ethnic groups, there are specific common founder mutations that account for the majority of GJB2-related hearing loss, for example, 35delG, 235delC, and R143W in the European, Japanese, and African populations, respectively.3 The relatively high carrier frequency of recessive GJB2 mutations residing in the human genetic pool suggests there may be phenotypic advantages counterbalancing NSHL. Examination of skin histology has revealed that individuals homozygous and heterozygous for the common African GJB2 mutation, R143W, had significantly thicker epidermis plus higher sodium and chloride sweat secretions than wildtype family members. Authors suggested these GJB2-associated epidermal phenotypes may provide a protective mechanism against pathogen invasion. Although clinically defined skin disease is not described with these or other NSHL alleles, it should be noted that specific dominant GJB2 mutations result in ectodermal disorders in which hyperkeratosis is a common feature.4 Here, we describe in vitro analysis of deafness-associated missense GJB2 mutations that provide further evidence of a physiological mechanism that could provide GJB2-phenotypic advantage in vivo.

these studies were also performed in fibroblasts with similar results (for example, M34T-Cx26 had an average of 40% more cells surviving than those cells transfected with WT-Cx26; n = 4 experiments), the reduced cell death phenotype may produce additional phenotypic advantages in other tissues. Surprisingly, the dominant NSHL mutation, T5M, in GJB6 encoding Cx30 also displayed reduced cell death compared to the wildtype protein. In contrast to GJB2, GJB6-associated NSHL mutations are very rare, suggesting that the different channel properties of Cx30 channels either homomeric or heteromeric may not confer the same selective advantage compared to Cx26-associated channels.

These data indicate a common trend of in vitro cell protection seen with all GJB2 NSHL-associated mutations tested. The phenotypic advantage gained from in vivo cell survival could vary across cell types counterbalanced against the disadvantage of deafness. Epidermal thickening is one advantage that has been assayed, whereas other more subtle functional advantages could also be gained in other tissues. Recently, the bacterium Shigella flexneri has been shown to induce the opening of Cx26 hemichannels providing evidence that pathogen-induced opening of Cx26 may promote bacterial invasion by promotion of signalling events.10 Interestingly, the proposed heterozygote advantage observed with another common recessive human disorder, cystic fibrosis (CF), is associated with restricting the invasion of Salmonella typhi into epithelial cells via the mutant CFTR chloride channel and thus providing protection against typhoid fever.11 Further studies will yield insights into the proposed heterozygote advantage associated with GJB2 mutations in different tissues. With respect to the skin, further investigations will be needed to assess if the epidermal thickening and the loss of functional Cx26 channels does indeed reduce bacterial invasion and infection rates in GJB2 heterozygotes.

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