LETTER TO JMG

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

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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.

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 NEB1 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 this population by PI fluorescence (fig 1). It was noted that subpopulations of NEB1 keratinocytes express Cx26 endogenously, as well as other epidermally expressed Cx isoforms including Cx30, Cx31, and Cx30.3. Little or no endogenous 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.

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 2) 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

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

Figure 1  FACS analysis of wildtype, hearing loss (NSHL) and skin disease-associated GJB2 mutations. EGFP expressing fusion proteins were studied for cell death after transfection (Transfast, Promega) of the NEB1 cell line in 60-mm culture dishes at 70% confluence (Corning). Culture media and adhered cells were harvested after 48 h to ensure all cells were FACS analysed. (A) FACS analysis scatter charts show the distribution of EGFP positive and negative cells in experiments with GJB2 wildtype, NSHL mutation R143W, and skin disease-associated mutation D50N constructs. EGFP positive cells are indicated in the boxed area and were used to measure cell death. (B) Plot charts show counts of 10 000 EGFP positive cells from the three corresponding scattercharts above. The intensity of propidium iodide uptake was used as an indicator of cell death and is shown as a percentage of the total EGFP expressing population. The NSHL mutation showed a reduction in percentage cell death compared to the wildtype construct. Massive levels of death were observed with the skin disease-associated mutation.

Figure 2  Histogram of transfected NEB1 cells FACS analysed in five replicate experiments. The transfection of deafness-associated mutant constructs M34T, W44C, and R143W in GJB2, and T5M in GJB6 resulted in a statistically significant reduction in cell death when compared to transfected wildtype plasmid constructs (as shown by t-test; *p<0.05). Cell death levels were normalised against cells expressing WT constructs to avoid background variation between individual experimental days and are shown as percentage change. The results were consistently replicated in all experiments conducted in NEB1 (n = 5 per construct).
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