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Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines

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Abstract

Alternative splicing is a biological mechanism that enables the synthesis of several isoforms with different or even opposite functions. This process must be tightly regulated to prevent unwanted isoform expression favoring pathological processes. Some isoforms of interleukin 32 (IL-32) are reported to be more potent in inducing inflammation, however the role in cell death remains to be investigated. This study demonstrates that IL-32γ and IL-32β can induce caspase-8-dependent cell death whereas this was not observed for IL-32α. Overexpression of IL-32β or IL-32γ but not IL-32α, resulted in enhanced expression of the survival cytokine IL-8. Furthermore, restoring the IL-8 signaling pathway by overexpressing CXCR1 in HEK293 cells, rescued IL-32β but not IL-32γ-induced cell death. Interestingly, IL-32γ was able to downregulate CXCR1 and thereby induce cell death. Subsequent studies into the role of IL-32 in thyroid cancer (TC) revealed that several IL-32 isoforms, IL-8, and CXCR1 are expressed in TC cell lines and specimens. Remarkably, TC cell lines were found to produce high concentrations of IL-8, indicating an important role for IL-8 in the survival-signaling pathway in these cells. Intriguingly, a significant correlation between the IL-8 receptor CXCR1 and IL-32γ was observed in TC specimens, while this was not observed for the other IL-32 splice variants. Blocking IL-32 alternative splicing by Isoginkgetin resulted in predominant expression of IL-32γ splice variants and cell death in TC cell lines. All together, modulation of IL-32 alternative splicing could represent a novel strategy for the treatment of malignancies, in particular thyroid cancer.

Introduction

Interleukin 32 (IL-32) is a proinflammatory cytokine (1–3) that can be expressed in several isoforms (3,4) generated through alternative splicing (5). The complete transcript that contains all exons is called IL-32γ and can be spliced into shorter isoforms such as IL-32β or IL-32α. Expression of IL-32 was reported in several autoimmune diseases such as rheumatoid arthritis (2,5–7), chronic obstructive pulmonary disease (1), inflammatory bowel disease (8) and atopic dermatitis (9). In addition, IL-32γ-transgenic mice displayed vessel abnormalities and enhanced IL-32 expression was reported in human atherosclerotic plaques (10), indicating an important role in the pathogenesis of cardiovascular diseases (10).

Besides autoimmune and inflammatory diseases, expression of IL-32 has been reported in different forms of cancer such as gastric cancer (11–15), lung cancer (16,17), breast cancer (18), colon cancer (19,20), renal cancer (21), esophageal cancer (22), liver cancer (23), pancreas cancer (24), non-medullary thyroid cancer (TC) (25) and leukemia (26,27). In these studies,

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Abbreviations

FAK-1	focal adhesion kinase 1
LDH	lactate dehydrogenase
IL	interleukin
SNP	single nucleotide polymorphism
TC	thyroid cancer

there are conflicting observations of either an oncogenic or a tumorsuppressive role for IL-32, which might originate from differences in the predominant IL-32 isoform expressed in the target tissue analyzed in every single study. One of these studies reported that IL-32 protein expression was the highest in gastric cancer followed by gastritis, both compared with normal stomach tissue (14). Unfortunately, IL-32 isoform expression has only been investigated in the gastritis samples and in the *Helicobacter pylori*-stimulated cells but not in the gastric cancer samples. Nevertheless, the authors reported that 90% of transcripts appeared to be IL-32β while IL-32α or IL-32γ was not detected (14).

We recently reported a single nucleotide polymorphism in the promoter region of IL-32 that enhanced LPS-induced IL-32γ (25). This IL-32 promoter single nucleotide polymorphism was overrepresented in TC patients and higher dosages of radioactive iodine treatment for successful tumor remission was required, indicating the important role of IL-32 in the etiology and development of therapy-resistance in TC (25). TC is the most prevalent endocrine malignancy. Unfortunately, treatment modalities for patients with advanced TC who no longer respond to conventional treatment with radioactive iodine are limited and novel therapies with multikinase inhibitors have been introduced but treatment responses are temporary and fail to cure the patient (28). Therefore, new treatment options are needed and inflammatory pathways have emerged as promising new targets for cancer therapy. Interest in IL-32 research is increasing and the number of reports linking IL-32 to cancer is rising (11,12,15–20,22–27,29–37). Although it is highly probable that IL-32 modulates the process of carcinogenesis, knowledge concerning alternative splicing of IL-32 and the features of the generated splice variants in terms of inducing cell death is lacking. It has been hypothesized that IL-32 has an important role in regulating cell survival and death. One of the first reports that demonstrated the relationship between IL-32 and cell death showed that apoptotic T-cells expressed more IL-32, while silencing of IL-32 rescued them (4). Furthermore, overexpression of IL-32γ appeared to be difficult due to cell death or cell arrest in human T-cells (4). In addition, intraarticular overexpression of IL-32γ induced chondrocyte death in an experimental mouse model (6). This capacity of IL-32 to induce cell death has been proposed as antitumor strategy, as IL-32γ-transgenic mice showed reduced tumor growth (35). However, other reports show protumorigenic properties for IL-32 such as promoting tumor cell proliferation, invasion, metastasis and angiogenesis (12,16,29,38).

The focus of this study is to decipher the function of IL-32α, IL-32β and IL-32γ isoforms in inducing cell death and how this can be utilized to develop novel anticancer strategies. For that purpose, we will investigate this in different tumor cell lines (HEK293, FTC133 and BC-PAP) and in tissue samples from patients with TC. By unraveling IL-32-induced cell death and how alternative splicing of IL-32 can be pharmacologically exploited to induce cell death in TC cells, a potential novel treatment strategy could be developed.

Materials and methods

Cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium-Glutamax medium (Gibco) with 10% fetal calf serum, pyruvate and penicillin–streptomycin. During transfection, HEK293 cells were maintained in RPMI1640 (Gibco) without phenol red but containing 5% fetal calf serum. FTC133 and BC-PAP cells were maintained in Dulbecco's modified Eagle's medium-Glutamax (Gibco) with 10% fetal calf serum, pyruvate and gentamycin (Gibco). Cells were cultured at 37°C with 5% carbon dioxide. HEK293 cells lack the expression of IL-8 receptor CXCR1 and were therefore authenticated by determining CXCR1 expression by qPCR. FTC133 and BC-PAP cell lines were directly obtained from Prof. Dr B. Haugen in 2012 and were previously characterized by using short tandem repeat and single nucleotide polymorphism array analysis (39). FTC133 and BC-PAP cell lines were resuscitated and used for in vitro experiments.

Expression vectors

PCDNA3-based expression vectors for expression of IL-32α, IL-32β and IL-32γ were generated by standard PCR and restriction based cloning methods. Expression plasmids were constructed with a Kozak sequence (5′-GCCGCCACC-3′) immediately upstream of the ATG start codon followed by the cDNA of full length human IL-32α, IL-32β, IL-32γ or eGFP (control plasmid). The CXCR1 plasmid (pORF9-hIL8RA) was commercially purchased (Invivogen, Toulouse, France).

Transfection, cell viability and cytotoxicity by IL-32α, IL-32β or IL-32γ

HEK293 cells were transfected with pCDNA3 plasmids expressing eGFP (control), IL-32α, IL-32β, IL-32γ or pORF9-hIL8RA (CXCR1). Briefly, 200 000 cells were seeded in 24-well plates (Corning Incorporated, Corning, NY, USA) and transfected by using lipofectamine 2000 (Invitrogen). Samples were taken at different time points (24, 48, and 72 h) and cytotoxicity was determined by trypan blue exclusion or with Cytotox96 assay (Promega, Madison, WI, USA). Western blot analysis was performed on the 24 h samples as described previously (6). Finally, a specific caspase-8 inhibitor (Z-IETD-FMK) (Biovision, Mountain View, CA, USA) was diluted 1000× to investigate IL-32-induced cell death.

Silencing of IL-32 in TC cell lines

A day before silencing, FTC133 and BC-PAP cells were seeded in 48-well plates containing 200 000 cells per well in RPMI1640 medium containing 5% fetal calf serum. The next day, siRNA transfection mix was prepared as follows; tube 1 contained 97.5 µl serum free RPMI1640 medium and 2.5 µl siRNA (10 µM stock) from Dharmacon (on-target Smartpool, control and IL-32). Subsequently, tube 2 contained 99.0 µl serum free RPMI1640 and 1.0 µl Dharmafect 1 (Dharmacon/Thermo Fisher Scientific, Waltham, MA, USA) and both tubes were mixed by gently pipetting up and down and incubated separately at room temperature for 5 min. After the first incubation, tube 1 was added to tube 2 and mixed gently by pipetting up and down followed by 20 min incubation at room temperature. Next, 800 µl of serum free RPMI1640 medium was added to the mixture and mixed by gently pipetting up and down. Finally, culture medium was discarded and transfection medium, 200 µl per well, was added and cells were placed in the incubator for 24 h. The following day, supernatants and RNA were isolated. Lactate dehydrogenase (LDH) release was measured by the Cytotox96 assay and IL-8 production by ELISA (R&D Systems, Minneapolis, MN, USA). IL-32 and IL-8 mRNA expression were determined as described in the next section.

RNA analysis by qPCR

Twenty-four hours after transfection Trizol (Invitrogen) was added to the cells to isolate RNA by an enhanced single-step method based on the method reported by Chomczynski and Sacchi (40). RNA was precipitated with isopropanol and washed with 75% ethanol followed by reconstitution in RNAse-free water. Subsequently, RNA was reverse transcribed into cDNA by using iScript (Bio-Rad, Hercules, CA, USA). Formalin fixed and paraffin embedded tissue derived from TC patients were anonymized and used to isolate RNA. Briefly,

several sections of the biopsies were incubated overnight with Proteinase K (Qiagen, CA, USA) followed by RNA-Bee extraction as described by the manufacturer (AMS Biotechnology, Abingdon, UK). Isolated RNA was transcribed into cDNA by using random hexamers (Promega) and qPCR analysis was done by using the ABI/PRISM 7000, 7300 and the StepOnePlus sequence detection systems with SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA). IL-32 isoform primers were developed as previously reported (5) and CXCR1, TNFα, IL-8 and IL-6 primer sequences were obtained from the Harvard Primerbank database and primers were purchased from Biolegio (Nijmegen, The Netherlands). The mRNA analysis was done with the 2^dCt method and normalized by using the housekeeping gene GAPDH.

Cytokine analysis by Luminex

In the supernatants of transfected cells, TNFα, IL-6 and IL-8 concentrations were determined by using the BioPlex system (Bio-Rad) together with the Milliplex cytokine kit (Millipore, MA, USA) at 24, 48 and 72 h.

Inhibition of alternative splicing by Isoginkgetin

The pre-mRNA splicing inhibitor Isoginkgetin (Merck Millipore, Billerica, MA, USA) was used to inhibit alternative splicing of IL-32. The inhibitor was dissolved in DMSO and two different concentrations were used, namely 10 and 30 μM. In control conditions, cells were treated with the same DMSO concentration as the Isoginkgetin exposed cells. Finally, caspase-8 activation was investigated in the Isoginkgetin-induced cytotoxicity by exposing the cells with Isoginkgetin and a specific caspase-8-inhibitor as previously described.

Results

Overexpression of IL-32β or IL-32γ in human HEK293 cells results in cell death mediated through caspase-8

To investigate the effects of the different isoforms of IL-32 on cell survival, IL-32 overexpression experiments in HEK293 cells were conducted. Western blot analysis showed IL-32 proteins smaller than 20 kDa when IL-32α was overexpressed while control transfected cells showed no IL-32 protein bands (Figure 1A). IL-32β or IL-32γ overexpression resulted in IL-32 proteins between 50 and 20 kDa (Figure 1A). Control- or IL-32α-transfected HEK293 cells induced no cell death, in contrast to IL-32β- or IL-32γ-transfected HEK293 cells (Figure 1B). The IL-32β- or IL-32γ-induced diminished cell viability was noticeable as early as 24 h post transfection and was more pronounced at 48 and 72 h (Figure 1B). Pharmacologically inhibition of caspase-8 activity reduced IL-32β- and IL-32γ-induced cell death (Figure 1C). These data demonstrate that enhanced expression of IL-32β or IL-32γ, but not IL-32α induce cell death.

Overexpression of IL-32β or IL-32γ in human HEK293 cells induces IL-8 synthesis

IL-32 has important effects not only on cell survival, but also on inflammation, with several proinflammatory cytokines being cell survival factors. Overexpression of IL-32γ in HEK293 cells significantly induced IL-8 mRNA expression compared with control transfection (Figure 2A). IL-6 or TNFα mRNA levels were not significantly changed by overexpression of IL-32α, IL-32β or IL-32γ (Supplementary Figure 1A and B, available at *Carcinogenesis* Online). Subsequently, IL-8, IL-6 and TNFα protein concentrations were measured in the culture supernatants at 24, 48 and 72 h. Overexpression of IL-32β or IL-32γ significantly enhanced IL-8 protein concentrations at all three time points compared with control transfection (Figure 2B). In contrast, overexpression of IL-32α did not result in increased IL-8 protein concentrations compared with control (Figure 2B). Furthermore, overexpression of IL-32α, IL-32β or IL-32γ did not result in different IL-6 and TNFα protein concentrations compared with control control (Supplementary Figure 1C and D, available at *Carcinogenesis* Online).

Restoring CXCR1 expression in human HEK293 cells rescues IL-32β- but not IL-32γ-induced cell death

IL-8 signaling is not only involved in chemotaxis but also in cell survival (41,42). Of note, HEK293 cells lack the IL-8 receptor CXCR1 and therefore are IL-8 unresponsive. Subsequently, expression of CXCR1 in HEK293 cells was restored by overexpression and

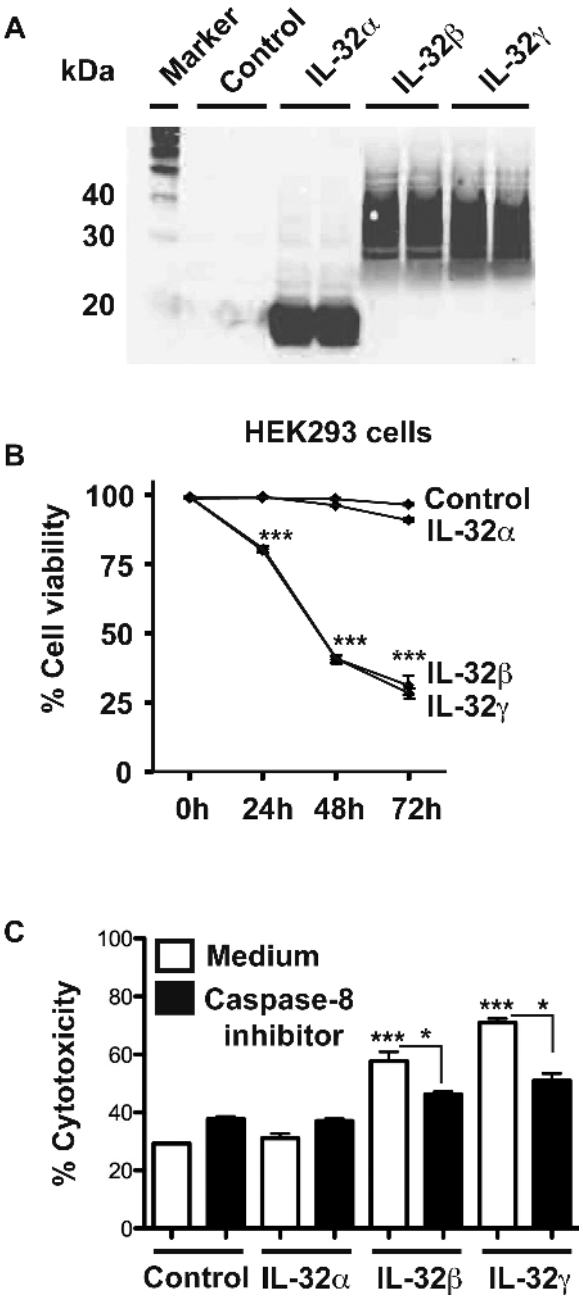

Figure 1. Overexpression of IL-32α, IL-32β or IL-32γ in human HEK293 cells. (**A**) Western blot analysis demonstrated that IL-32 proteins were expressed in the human HEK293 cells with different sizes while control transfected cells showed no IL-32 protein expression. (**B**) At 24, 48 and 72 h post-transfection the cell viability was assessed by trypan blue exclusion and overexpression of IL-32β or IL-32γ showed decreased cell viability. (**C**) A specific caspase-8 inhibitor was able to reduce the IL-32β- and the IL-32γ-induced cytotoxicity. Results expressed as mean ± SEM, One-way ANOVA with Dunnett's multiple comparison test was used for comparing control versus experimental groups and Mann–Whitney *U* test was used to test significant differences between medium and caspase-8 inhibitor, $n = 4$, $^{*}P < 0.05$, $^{***}P < 0.001$.

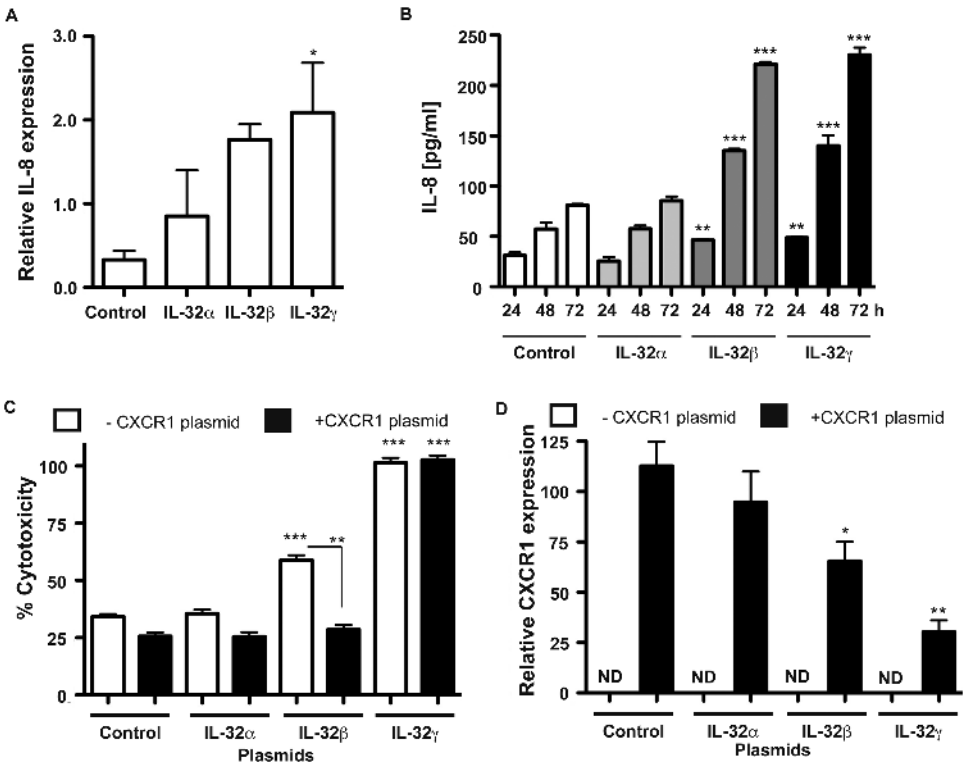

Figure 2. Restoring the IL-8 signaling pathway in human HEK293 cells prevented IL-32β- but not IL-32γ-induced cytotoxicity. (**A**) Induction of IL-8 mRNA was observed after IL-32β or IL-32γ overexpression in human HEK293 cells 24 h post-transfection. (**B**) Significant production and secretion of IL-8 was observed after IL-32β or IL-32γ overexpression in human HEK293 cells at 24, 48 and 72 h post transfection. (**C**) Overexpression of IL-32β or IL-32γ resulted again in enhanced cytotoxicity after while restoring the IL-8 signaling pathway by overexpression of CXCR1 rescued the IL-32β- but not the IL-32γ-induced cytotoxicity. (**D**) Expression of CXCR1 was significantly reduced after IL-32β but even more after IL-32γ overexpression while HEK293 cells did not expressed CXCR1. Results expressed as mean ± SEM, One-way ANOVA with Dunnett's multiple comparison test was used for comparing control versus experimental groups and Mann–Whitney *U* test was used to test differences between with or without CXCR1 overexpression, $n = 4$ (A, B), $n = 6$ (C), $n = 3$ (D), time points post-transfection: 24 h (A), 24–48–72 h (B), 48 h (C, D), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the consequences for IL-32-induced cell death were investigated. As previously observed, overexpression of IL-32β or IL-32γ induced cell death but co-overexpression with CXCR1 rescued IL-32β- but not IL-32γ-induced cell death (Figure 2C). A plausible explanation could be that IL-32γ downregulated CXCR1 mRNA expression. Accordingly, the CXCR1 expression was severely reduced by overexpression of IL-32γ (Figure 2D) whereas this was less for IL-32β and not significant for IL-32α overexpression. As expected, HEK293 cells did not express CXCR1 (Figure 2D), as reported in literature (43,44).

IL-32, IL-8 and CXCR1 expression in human TC cell lines

In order to assess the relevance of these findings for TC, we studied IL-32 in TC cell lines. Expression of IL-32α, IL-32β and IL-32γ was detected in both the human follicular TC cell line FTC133 (PTEN deficient) and in the human papillary TC cell line BC-PAP (BRAF V600E mutated). IL-32α and IL-32γ mRNA expression was comparable between both cell lines, whereas expression of IL-32β was significantly higher in BC-PAP cells (Figure 3A), which lead to concomitant differences in ratios of splice variants between cell lines (Figure 3B). IL-8 expression was significantly higher in BC-PAP cells, whereas CXCR1 expression was comparable between the two cell lines (Figure 3C–E). Finally, significant correlations were observed between the expression of IL-32β and IL-8, IL-32β and CXCR1, IL-32γ and IL-8 and IL-32γ and CXCR1 in BC-PAP cells (Supplementary Table 1, available at *Carcinogenesis* Online). Remarkably, an almost complete correlation was observed between the expression of IL-32γ and CXCR1 in the BC-PAP cell line (Pearson's r of 0.9957).

Silencing of IL-32 in human TC cell lines

IL-32 was silenced in FTC133 and BC-PAP cells and analysis of IL-32 mRNA expression demonstrated that silencing was more efficient in FTC133 compared with BC-PAP cells (Figure 3F and I). Nevertheless, significant reduction in IL-8 and LDH secretion was observed in both cell lines (Figure 3G, H, J, K).

Correlation between IL-32, IL-8 and CXCR1 in thyroid biopsies from patients with various thyroid diseases

25 human TC tissues specimens (Supplementary Table 2, available at *Carcinogenesis* Online) were investigated for IL-32α, IL-32β, IL-32γ, IL-8 and CXCR1 mRNA expression and correlation studies were performed. IL-32α expression was detected only in half of the samples, whereas expression of IL-32β, IL-32γ, IL-8 and CXCR1 were detected in all biopsies. IL-32α expression did not correlate with IL-8 or CXCR1 expression (Figure 4A and B) and this was also the case for IL-32β (Figure 4C and D). IL-32γ mRNA expression correlated significantly with the mRNA expression of CXCR1 but not with IL-8 (Figure 4E and F).

Inducing cell death in thyroid cancer cell lines by inhibition of alternative splicing

TC cell lines FTC133 and BC-PAP were exposed to two different concentrations of Isoginkgetin to inhibit the IL-32 alternative

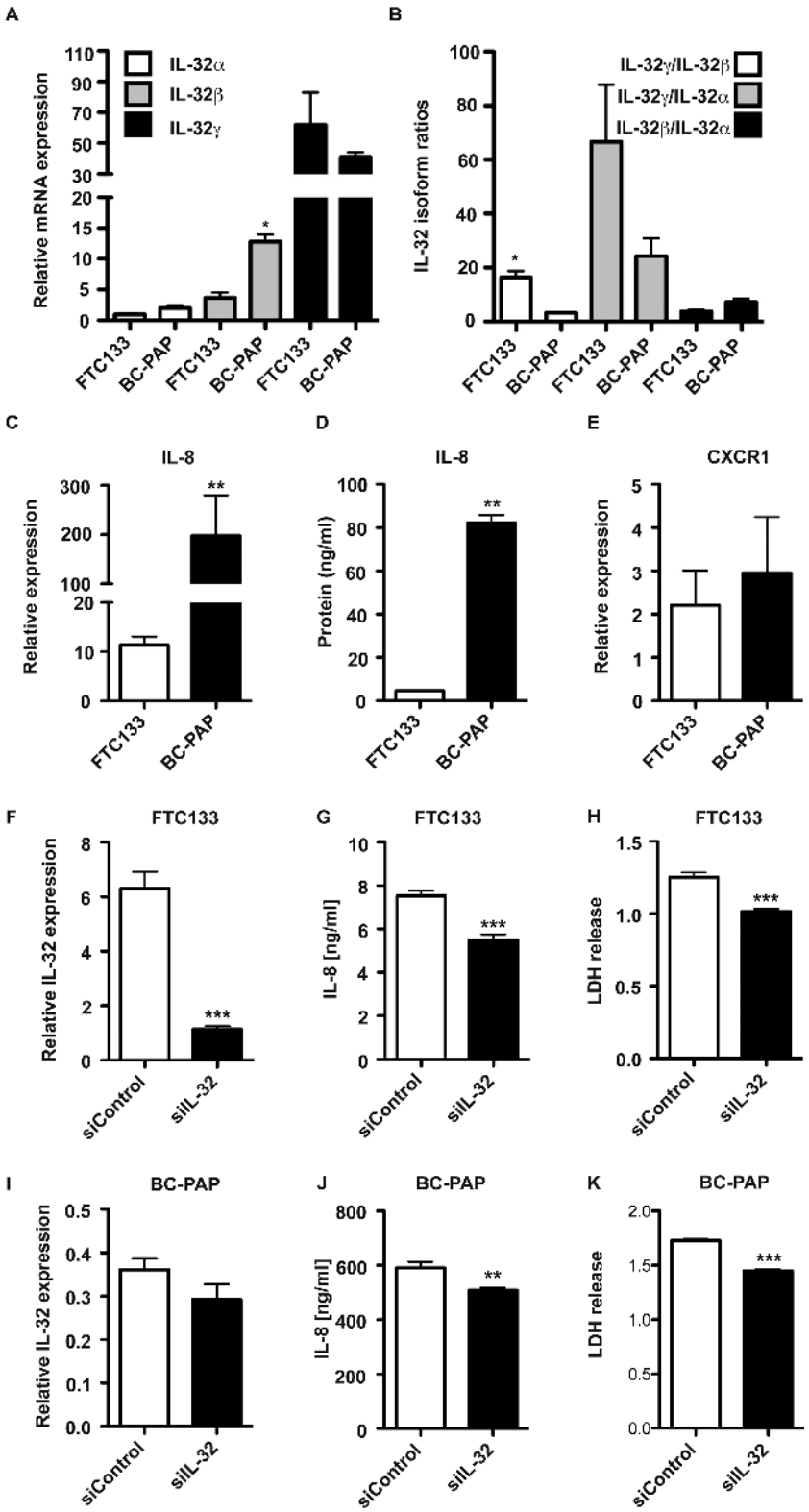

Figure 3. IL-32, IL-8, CXCR1 expression and IL-32 knockdown in thyroid cancer cell lines. (**A**) Expression of IL-32α, IL-32β or IL-32γ mRNA in human FTC133 and BC-PAP cells. (**B**) IL-32 splicing ratios in human FTC133 and BC-PAP cells. (**C**) Relative IL-8 mRNA expression in human FTC133 and BC-PAP cells. (**D**) Production of IL-8 in supernatants of human FTC133 and BC-PAP cells. (**E**) Expression of CXCR1 mRNA in human FTC133 and BC-PAP cells. (**F**) Relative IL-32 mRNA expression after silencing of IL-32 in FTC133 cells. (**G**) Reduction in IL-8 production and secretion after IL-32 knockdown in FTC133 cells. (**H**) Reduction in LDH release after IL-32 silencing in FTC133 cells. (**I**) Relative IL-32 mRNA expression after silencing of IL-32 in BC-PAP cells. (**J**) Reduction in IL-8 production and secretion after IL-32 knockdown in BC-PAP cells. (**K**) Reduction in LDH release after IL-32 silencing in BC-PAP cells. Results expressed as mean ± SEM at 24 h, Mann–Whitney *U* test, relative IL-32 expression and ratios $n = 4$, IL-8 mRNA/protein and CXCR1 mRNA $n = 6$, IL-32 silencing $n = 6$–12, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

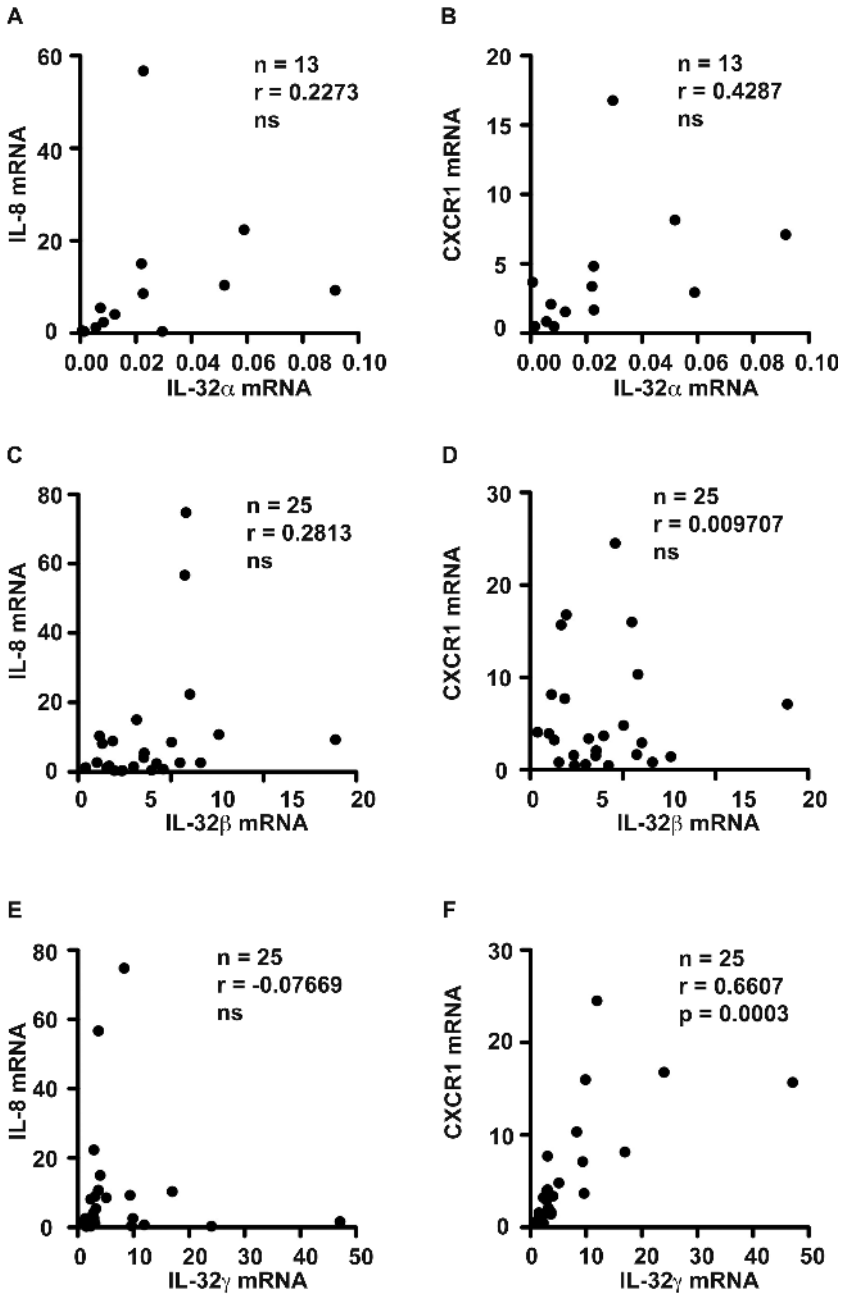

Figure 4. IL-32 splice variants correlated with IL-8 or CXCR1 in human thyroid cancer specimens. (**A**) Correlation between IL-32α and IL-8; (**B**) IL-32α and CXCR1; (**C**) IL-32β and IL-8; (**D**) IL-32β and CXCR1; (**E**) IL-32γ and IL-8; (**F**) IL-32γ and CXCR1. Results expressed as individual dots and tested by Pearson's correlation test.

splicing and to induce cell death. Incubation with the highest concentration skewed IL-32 alternative splicing towards IL-32γ in both FTC133 and BC-PAP cells (Figure 5A and B). BC-PAP cells displayed a reduction in IL-32β splice variants with both concentrations of Isoginkgetin (Figure 6B). Cytotoxicity as measured by LDH release was highest in the FTC133 cell line with the 30 μM Isoginkgetin concentration (Figure 5A). Although cytotoxicity in the BC-PAP cells was lower than in FTC133, it was still highly significant compared with control (Figure 5B). Cell death morphology was clearly visible in the FTC133 cells but less obvious in the BC-PAP cells (Figure 5C). Because cytotoxicity was highest in the FTC133 cells exposed to 30 μM Isoginkgetin, blocking of caspase-8 was investigated in those conditions and showed that cytotoxicity could significantly be reduced (Figure 5D).

Discussion

In this study, overexpression of IL-32β or IL-32γ induced IL-8 and caspase-8-dependent cell death in human HEK293 cells. In contrast, control or IL-32α-transfected HEK293 cells showed no reduced cell viability or IL-8 production. The amount of IL-32β/IL-32γ-induced IL-8 was in the pg/ml range in the HEK293 cells, while TC cells spontaneously produce ng/ml of IL-8, indicating that the IL-8 survival-signaling pathway is essential in TC. Of note, HEK293 cells lack the receptors for IL-8 signaling and restoring CXCR1 expression consequently might prevent IL-32β/IL-32γ-induced cell death. IL-32β-induced cell death could indeed be rescued through restoring the IL-8 signaling in HEK293 cells, demonstrating that IL-8 signaling is pivotal for preventing

IL-32β-induced cell death. However, the IL-32γ-induced cell death could not be rescued by overexpressing CXCR1, since it appeared that IL-32γ downregulated CXCR1 overexpression. One important question is whether intracellular or extracellular IL-32 is responsible for the induced cell death and induction of IL-8. As reported previously by us, most IL-32 proteins will stay intracellular (5,45,46) but during inflammation and/or when splicing of the longest isoform (IL-32γ) is prevented, non-spliced IL-32γ can be efficiently secreted (5). Besides non-spliced IL-32γ secretion, the process of cell death will likely release IL-32 proteins in the extracellular compartment and potentially binds to cell surface receptors and evoke cell signaling. A typical cytokine receptor was not discovered for IL-32 but we reported that IL-32 could bind to the extracellular part of αVβ3 and αVβ6 integrins (47) and might act as receptors through focal adhesion kinase 1 (FAK-1) signaling. Recently, we reported that extracellular recombinant IL-32γ could induce angiogenesis in a dose dependent manner and that IL-32γ-induced angiogenesis could be inhibited by an αVβ3 blocker (38), demonstrating that extracellular recombinant IL-32 could indeed signal via αVβ3 integrins. Furthermore, we reported that human umbilical vein endothelial cells (HUVECs) stimulated with extracellular recombinant IL-32γ showed no

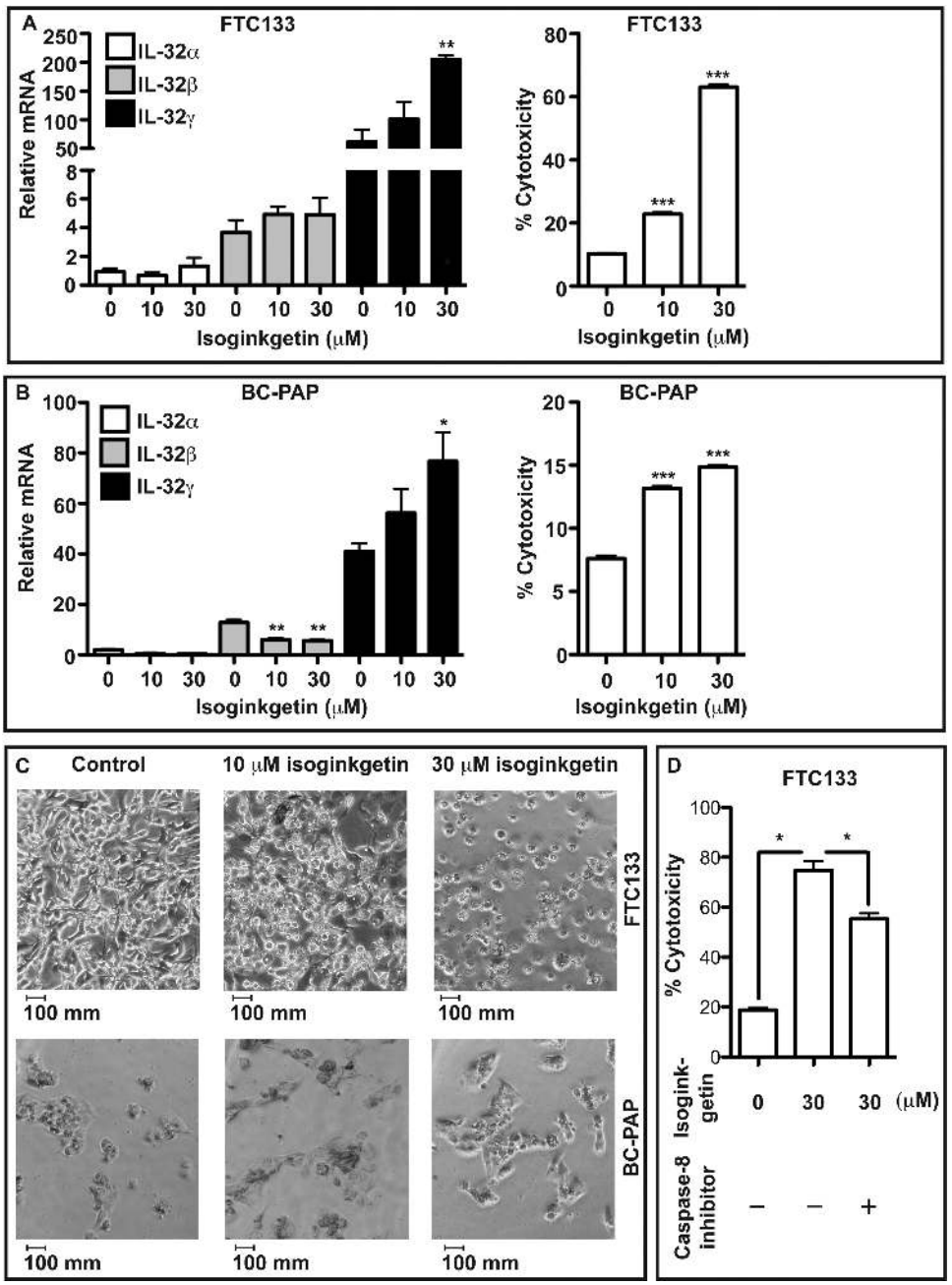

Figure 5. Induction of cell death through inhibition of alternative splicing in human thyroid cancer cells via caspase-8. (**A**) Exposure to Isoginkgetin induced IL-32γ and enhanced cytotoxicity in human FTC133 cells. (**B**) Induction of IL-32γ, reduction of IL-32β and enhanced cytotoxicity after treatment with Isoginkgetin in human BC-PAP cells. (**C**) Cell death morphology observed in human FTC133 but less obvious in human BC-PAP cells. (**D**) Isoginkgetin-induced cytotoxicity was significantly reduced in FTC133 cells after blocking caspase-8. Results expressed as mean ± SEM 48 h post treatment with Isoginkgetin, One-way ANOVA with Dunnett's multiple comparison test (A, B, C) was used for comparing control versus experimental groups, Mann–Whitney *U* test (D), $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

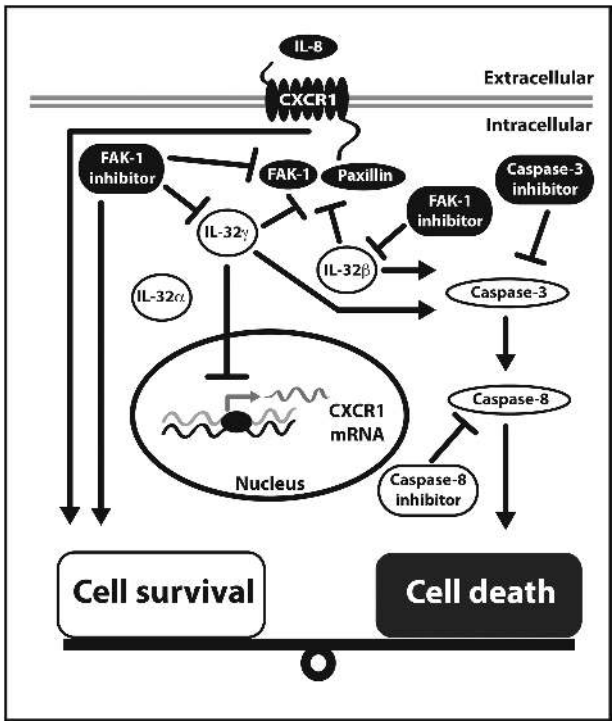

Figure 6. IL-32-induced cell death; activation of FAK-1 and Paxillin through CXCR1/IL-8 activation leads to cell survival. IL-32γ and IL-32β but not IL-32α induce cell death through binding to FAK-1/Paxillin that leads to activation of caspase-3 and subsequently caspase-8. Interfering with the IL-32/FAK-1 binding using a specific FAK-1-inhibitor that significantly reduced the IL-32γ/IL32β-induced cytotoxicity. Interestingly, blocking the caspase pathway could reduce cell death and finally, IL-32γ could reduce the transcription of CXCR1 mRNA and thereby antagonize the IL-8 survival signaling. These findings demonstrate that IL-32 is a key player in tumor survival and consequently a promising target in the cancer.

differences in expression of genes involved in apoptosis (38), indicating that cell death probably is not induced by extracellular IL-32. Additionally, we reported that IL-32 could bind to FAK-1 and paxillin (47), demonstrating that IL-32 is able to bind intracellular proteins besides αVβ3 and αVβ6 integrins. Both FAK-1 and paxillin are important for cell survival signaling and intracellular IL-32 might block this. On the other hand, induction of IL-8 might be due to extracellular IL-32 as we reported that by blocking αVβ3, IL-8 induction by recombinant IL-32γ was dramatically reduced (38). All together, intracellular IL-32 is responsible for the induction of cell death rather then extracellular IL-32 and IL-8 production is possibly induced by extracellular IL-32.

In the TC cell lines used in this study, we found mRNA expression of IL-32α, IL-32β, and IL-32γ. IL-32β expression was significantly higher in the human papillary TC cell line BC-PAP compared with the human follicular TC cell line FTC133, while IL-32γ expression was comparable between the two cell lines. Moreover, by analyzing the IL-32 isoform ratios it became clear that IL-32γ to IL-32β splicing was significantly lower in FTC133 cells compared to BC-PAP cells, and this implies that FTC133 cells contain relatively more IL-32γ due to less splicing activity. This was also observed for IL-32γ to IL-32α splicing, although the differences were not significant. Furthermore, IL-8 production was significantly lower in FTC133 cells compared with BC-PAP cells while CXCR1 expression was comparable, suggesting that the IL-8 signaling pathway is more active in BC-PAP cells. The three different IL-32 isoforms did not correlate with IL-8 or CXCR1 expression in the FTC133 cells, but significant correlations were observed in the BC-PAP cells between the expression of IL-32β and IL-8, IL-32β and CXCR1, IL-32γ and IL-8, and between IL-32γ and CXCR1. Intriguingly, a significant correlation between the IL-8 receptor CXCR1 and IL-32γ was observed in TC specimens, while this was not observed for the other IL-32 splice variants. Apparently, IL-32γ correlates very strong with CXCR1 because (i) IL-32γ was able to downregulate CXCR1, (ii) an almost perfect correlations in BC-PAP cells was observed (Pearson's r of 0.9957), (iii) finally correlation between IL-32γ and CXCR1 in patients with various thyroid diseases. These data suggest that the IL-32/IL-8 pathway plays an important role in modulation of cell death/ cell survival in TC. To explore this further, IL-32 expression was silenced in FTC133 and BC-PAP cells and silencing was more efficient in FTC133 cells. Consequently, silencing of IL-32 resulted in lower IL-8 production and less LDH release into the supernatants that reflex leaky cells/cell death. This means that IL-32 functions as key regulator of cell death because overexpression drives cell death and IL-8 production, while silencing could prevent this.

Modulation of IL-32 splicing was achieved by using the natural compound Isoginkgetin and was studied in the two TC cell lines. When we applied Isoginkgetin to the TC cell lines, we found a significantly increased IL-32γ expression in the FTC133 and BC-PAP cell lines as well. Interestingly however, in the BC-PAP cells the amount of IL-32β transcripts reduced, while IL-32γ transcripts increased, which indicates that indeed alternative splicing from IL-32γ to IL-32β is inhibited. Induction of cell death was clearly observed in the FTC133 cells and in the BC-PAP cells although cytotoxicity was more obvious in the FTC133 cells. This is consistent with the finding of a more active IL-8 survival pathway in the BC-PAP cells as shown above that might provide the BC-PAP cells with a survival benefit, rendering them less sensitive to treatment with the splicing inhibitor. Subsequently, cytotoxicity was further investigated in FTC133 cells and blocking of caspase-8 significantly reduced the Isoginkgetin-induced cell death.

Alternative splicing is a powerful regulator that controls which isoforms are being generated in certain tissues and pathological conditions. These splice variants can display similar, different or even opposite functions. Two examples of how alternative splicing can change protein function are reported for IL-6 and IL-24. One of the properties of IL-6 is to promote cell proliferation, however an alternatively spliced IL-6 variant can block this (48). Similarly, IL-24-induced cell death can be antagonized by an IL-24 splice variant (49). In this study, we demonstrated that IL-32 isoforms have distinct functions: (i) IL-32γ and IL-32β, but not IL-32α, induce cell death, (ii) cell death induced by IL-32β can be rescued through the IL-8 survival-signaling pathway and (iii) IL-32γ is the most potent inducer of cell death due to its capacity to block the IL-8 survival-signaling pathway. In TC cell lines, blocking IL-32 alternative splicing resulted into a shift toward IL-32 splice variants and caused cell death and therefore, modulation of IL-32 alternative splicing could be explored as potential novel treatment strategy in patients with advanced TC.

Supplementary material

Supplementary Tables 1 and 2 and Figure 1 can be found at http://carcin.oxfordjournals.org/

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