Induction of apoptosis by human amylin in RINm5F islet β-cells is associated with enhanced expression of p53 and p21$^{WAF1/CIP1}$

Shaoping Zhang[a], Junxi Liu[a], 'Etuate L. Saafi[a], Garth J.S. Cooper[a,b,*]

[a] *The School of Biological Sciences, Level 4, University of Auckland, Auckland, New Zealand*
[b] *The Department of Medicine, School of Medicine, University of Auckland, Auckland, New Zealand*

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Abstract Human amylin (10 μM) significantly inhibited RINm5F islet β-cell proliferation and evoked apoptosis associated with typical degenerative ultrastructural changes and DNA fragmentation, whereas rat amylin did not. Time course analysis showed that human amylin elicited apoptosis in a passage-dependent manner. Expression of the apoptosis-related genes *p53*, *bcl-2* and *WAF1/CIP1* was examined using Northern blots. mRNAs corresponding to p53 and to p21$^{WAF/CIP1}$ were remarkably increased following human amylin treatment, whereas no change in bcl-2 was detected. Our data suggest a role of p53 and p21 in human amylin-induced β-cell apoptosis. Furthermore, cells with higher proliferative potential (lower passage) were found to be more susceptible to apoptosis and to induction of p53, suggesting that β-cells with different proliferation rates respond differently to human amylin, and that human amylin may be more toxic to proliferating cells.

Key words: Human amylin; Islet β-cell; Apoptosis; p53; p21$^{WAF1/CIP1}$

1. Introduction

Amylin (also termed islet amyloid polypeptide, IAPP) is a 37-amino acid peptide, which is produced by pancreatic islet β-cells and co-secreted with insulin in response to nutrient stimuli [1,2]. Studies have demonstrated that amylin is the key protein component of amyloid deposits found in the islets of Langerhans in almost all patients with non-insulin-dependent diabetes mellitus (NIDDM) [1,3]. A particular region of the peptide between positions 20 and 29, termed the amyloidogenic sequence, is thought to be responsible for amyloid fibril formation by the human peptide [4,5]. The sequence of human amylin is identical in those with NIDDM and non-affected individuals, and the mechanism of amyloid deposition in diabetes remains unclear at present. Furthermore, islet amyloid formation is not found in diabetic rats, whose amylin molecule has a different amino acid sequence in this molecular region [6]. Evidence suggests that amyloid formation may be related to hypersecretion of amylin associated with hyperglycemia acting in conjunction with other unknown factors which may enhance pancreatic fibril formation [7,8].

Possible roles for amylin in the pathogenesis of diabetes have been suggested [6,9,10]. Amylin has been implicated in insulin resistance and abnormal insulin secretion, both of which are characteristic of NIDDM [11,12]. The inhibitory effects of amylin on glycogen synthesis in isolated skeletal muscles have also been reported [13,14]. In addition, a number of studies have suggested that human amylin may be toxic to islet β-cells [10,15–17]. Ultra-structural analysis has indicated that human amylin spontaneously aggregates into a β pleated-sheet fibril structure in vitro, whereas no such ordered structure has been observed for rat amylin [18,19]. It has been suggested that contact of these fibrils with β-cell membranes is an event that can induce apoptosis [10,16], and that human amylin may exert a direct toxic effect on the pancreatic β-cell. This toxic effect of human amylin involves RNA and protein synthesis [10]. Other studies have suggested that intermediate-sized toxic amyloid particles, rather than mature amyloid deposits containing large (>5 nm diameter) fibrils, constitute the toxic form of matter [16]. Furthermore, over-expression or excessive secretion of human amylin may result in raised local and/or circulating concentrations. Such an elevation in human amylin concentrations could promote amyloid formation inside and/or outside β-cells [20,21]. Indeed, onset of diabetes, associated with islet amyloid formation and decreased β-cell mass, has been demonstrated in transgenic mice expressing human amylin in their β-cells [7,8,22]. Over-expression of human amylin but not rat amylin in COS-1 cells also resulted in intracellular amyloid formation which was associated with cell death [23,24]. In the light of these observations, human amylin could play a significant role in the progressive loss of β-cells in NIDDM.

The underlying molecular mechanism by which human amylin evokes β-cell death is not yet known. The evidence reviewed above has suggested that apoptosis could be the mode of cell death induced by human amylin in NIDDM. Apoptosis is an active cell death process characterized by cytoplasmic blebbing, apoptotic body formation, chromatin condensation, breakup of the nucleus and DNA fragmentation [25]. A number of genes, including cellular oncogenes c-*myc*, *bcl-2*, c-*fos*, c-*jun* and the tumor suppressor gene *p53*, have been implicated in the control of apoptosis in mammalian systems [25–27]. The tumor suppressor gene *p53* encodes a nuclear phosphoprotein that has been shown to play a critical role in regulating cell proliferation, the G1 cell cycle checkpoint and apoptosis [28,29]. Over-expression of wild-type p53 often leads to induction of cell cycle arrest and/or apoptosis in a variety of cell types [26,30]. In response to DNA damage or other forms of cellular injury, increased expression of p53 activates downstream target genes, such as p21$^{WAF1/CIP1}$ (p21), a cyclin-dependent kinase inhibitor [28,29]. p21 inhibits kinase activity of various cyclin/cyclin-dependent kinase complexes, which are key elements in cell cycle progression, thereby preventing the transition from the G1 to the S phase of the cell cycle and allowing time for repair of damaged DNA [26,28]. Alternatively, p53 may repress the

*Corresponding author. Fax: (64) (9) 373 7045.
E-mail: g.cooper@auckland.ac.nz

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bcl-2 (B-cell lymphoma/leukemia) oncogene, another downstream effector in the p53-dependent pathway of growth control [28]. Bcl-2 has been shown to inhibit apoptotic processes in some cell systems [27,28,31]. Expression of bcl-2 can prevent death of neurons deprived of nerve growth factor [31]. On the other hand, it has been shown that bcl-2 does not protect against cytotoxic T-cell killing [32]. Therefore, bcl-2 cannot protect every type of cell against apoptosis.

The aim of this study was to better understand the mechanism by which human amylin evokes death in islet β-cells. Here, we investigated the time course of induction of apoptosis by human amylin in a rat insulinoma cell line, RINm5F, to determine whether any known apoptosis-related genes could be implicated in this process.

2. Materials and methods

2.1. Cell culture and peptide treatment

RINm5F cells were cultured at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, 290 mg/ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 mg/ml sodium hydrogen carbonate as described [33]. For amylin treatment, stock solutions (500 μM) were prepared by dissolving synthetic human amylin (Lot ZL934, Bachem, CA) or rat amylin (Lot ZM275, Bachem, CA) powder in water and incubating at room temperature for 10 min. Cells (passage 26 and 53) were cultured in 75 ml flasks to approximately 70% confluence, after which human or rat amylin was added to a final concentration of 10 μM. The cultures were then incubated for further periods of 1, 2, 4, 8, 16 or 24 h, respectively. Untreated control cells were also cultured and processed in an otherwise equivalent manner. For subsequent experiments, cells were harvested by trypsinization and centrifugation.

2.2. Determination of cell proliferation rate

RINm5F cells (1×10^5) from passage 26 were seeded in 12 well plates and cultured for 24 h. The cultures were then divided into three groups: the first group (control) was cultured in the absence of amylin, the second group was cultured in the continuous presence of 10 μM human amylin, whereas the third group was cultured in the continuous presence of 10 μM rat amylin. Cells were then harvested every 24 h by trypsinization and cell numbers counted with a hemocytometer. Six cultures were used for experiments at each time point.

2.3. SEM analysis

The human amylin stock solution was incubated at room temperature for 1 h to allow fibril formation. RINm5F cells (passage 35) were cultured at 37°C on 13 mm diameter glass coverslips (Deckgläser, West Germany) placed on the bottom of 24 well culture plates (Linbro) until 80% confluence, after which human amylin stock solution was added to give a final concentration of 10 μM; an equivalent amount of water was added to control wells. Cells were further grown at 37°C for 22 h and then fixed for 3 h at room temperature with 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2), following which they were washed with 0.1 M Sorensen's buffer (pH 7.2) for 3×10 min, then post-fixed with 0.1% osmium tetroxide/0.1 M Sorensen's phosphate buffer (pH 7.2) for 1 h at room temperature. Cells were then serially dehydrated for 1×10 min with 30%, 50%, 70% and 90% ethanol, then 2×10 min with 100% ethanol, after which they were critical point dried before specimen coverslips were mounted onto SEM stubs. The specimens were viewed using a Phillips SEM 505 model scanning electron microscope.

2.4. Analysis of DNA fragmentation

RINm5F cell cultures were exposed to human or rat amylin for the time periods described above. Genomic DNA was extracted with DNAzol reagent (Gibco BRL) using a modification of the manufacturer's protocol. Cells were digested in 2 ml DNAzol reagent for 10 min at room temperature, followed by phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extractions. DNA was then precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA. 5 μg of each DNA sample was subjected to electrophoresis in a 1.2% agarose gel containing 0.1 μg/ml ethidium bromide. The DNA was then visualized under UV light. A 100 bp DNA ladder (Gibco BRL) was run simultaneously to serve as a size marker.

2.5. Quantitative Northern blot analysis

RINm5F cell cultures were exposed to human or rat amylin for various times as described above. Total RNA was isolated using an RNeasy Midi kit (Qiagen). Northern blots were performed using standard techniques as described [34]. 25 μg of each RNA sample was used for electrophoresis in a denaturing 1% agarose-formaldehyde gel, then transferred onto a Hybond N^+ nylon membrane (Amersham). The membrane was then hybridized with various probes: p53, p21 or bcl-2, respectively. Probes were labelled with [α-^{32}P]dCTP (Amersham) using a random primer labelling system (Gibco BRL). Hybridizations were carried out at 65°C for 18 h. The membranes were washed at room temperature in $2\times$SSC for 30 min, then at 65°C in $1\times$SSC for 15 min. mRNA levels were quantified using a phosphor imager (BAS 2040, Fuji, Japan). All membranes were later stripped and rehybridized with a GAPDH probe to normalize mRNA levels. Three independent experiments were performed for each RNA hybridization.

The cDNA probe for p53 was kindly provided by Dr. Evelyne May, Laboratoire d'Oncologie Moléculaire, IRSC-CNRS, Villejuif Cedex, France. The cDNA probes for p21 and bcl-2 were generated by RT-PCR using the following primers: p21, 5′-TTGCGATGCGCTCATGGCGAGC-3′ and 5′-CGACAAGGCCACGTGGTCCTCC-3′, amplifying a 248 bp fragment; bcl-2, 5′-GTGGCCTTCTTTGAGTTCGGTGG-3′ and 5′-AGGTCTGCTGACCTCACTTGTGG-3′, amplifying a 292 bp fragment.

3. Results and discussion

3.1. Effect of human amylin on RINm5F cell proliferation and apoptosis

In order to understand the potential role of human amylin in the induction of apoptosis and its possible contribution to islet β-cell loss in NIDDM, the inhibitory effect of human amylin on cell proliferation was studied in RINm5F cells, which are widely used as a model for functional studies of pancreatic islet β-cells [35]. As shown in Fig. 1, cells (passage 26) were cultured in the presence or absence of human or rat amylin and cell numbers determined over three days. RINm5F cell proliferation was markedly inhibited by 10 μM human amylin. In the absence of peptide, the number of control cells doubled approximately every 24 h in RPMI 1640 medium supplemented with 10% fetal calf serum. By contrast, in the continuous presence of 10 μM human amylin,

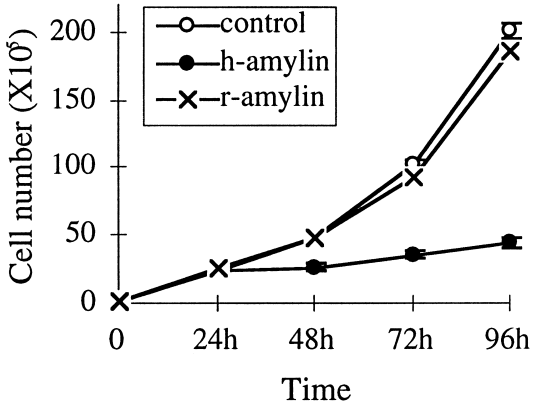

Fig. 1. Effect of human amylin on RINm5F cell proliferation. Cells (1×10^5) from passage 26 were seeded and cultured in 12-well plates. From 24 h onwards, cells were incubated in the absence (○) or presence of 10 μM human (●) or rat (×) amylin and cell numbers determined at the time points indicated. Data represent mean ± S.E.M. of six replicates at each time point.

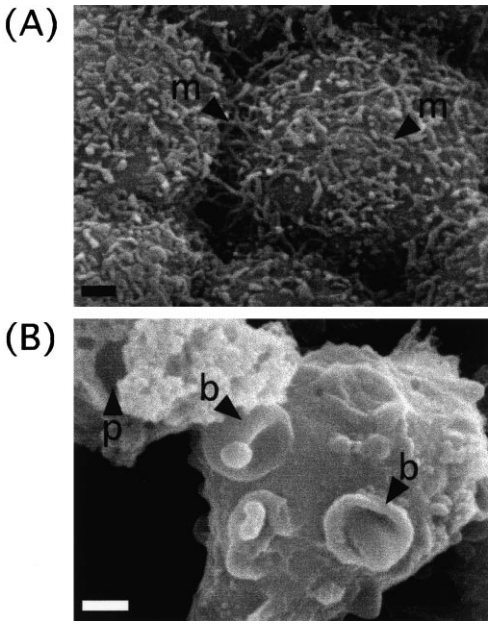

Fig. 2. Scanning electron micrographs of RINm5F cells exposed to human amylin. A: Untreated control cells with the arrows indicating the relative abundance of microvilli (m) on the cell surface. B: Otherwise identically treated RINm5F cells. Arrows indicate blebs (b) and pits (p) on the cell surface. Scale bars = 1 μm.

the number of cells increased by only about 10% after 24 h. Interestingly, cells exposed to rat amylin retain a proliferative capacity equivalent to that of untreated controls, indicating that this peptide has little effect compared with human amylin.

Rat amylin differs from the human peptide at six residues, five of which lie within the 'amyloidogenic' region, amylin$^{20-29}$ [6,36]. Circular dichroism studies suggested that human amylin adopts highly ordered stacking of β-sheet structures in solution, whereas, by contrast, rat amylin demonstrated no detectable β-structure in several solvents [37]. Aqueous solutions of synthetic human amylin spontaneously form polymorphic fibrils [5,19]. Recent studies with time-lapse atomic force microscopy have shown that these solutions can generate a previously uncharacterized protofibril, 2.4 nm in diameter [38], which is likely to serve as the elemental form underlying higher order assemblies containing coiled or ribbon-like fibrillar structures [19]. Our current data, taken together with those of others [10], suggest that a form of matter associated with the propensity of human amylin to form aggregated states containing β-pleated sheet structures is likely to underlie its ability to inhibit β-cell proliferation. However, currently available data do not exclude the possibility that sequence variations between human and rat amylin not associated with their different tendencies to fibril formation might also contribute to their different effects on cell proliferation.

The observed smaller increase in cell population in human amylin-treated cells in comparison to controls may result from either a decline in the proliferation rate or an increase in cell death, or both. To investigate the mechanism, RINm5F cells were treated with 10 μM human amylin for 22 h, then subjected to morphological analysis using scanning electron microscopy. The results, which are shown in Fig. 2, revealed typical features common to cells undergoing apoptosis. We observed the loss of microvilli from the cell surface and its eventual smoothening, and the formation of blebs and pits on the cell surface (Fig. 2B). These observations suggest that RINm5F cells exposed to human amylin were undergoing apoptosis. Under the same treatment conditions, apoptosis of RINm5F cells has also been demonstrated by means of the TUNEL (TdT-mediated dUTP-X nick end labelling) reaction (G. MacGibbon and G.J.S. Cooper, unpublished data). Furthermore, time course studies showed that induction of apoptosis triggered by human amylin was also accompanied by DNA fragmentation (Fig. 3). Fragmented DNA, which appears as a series of bands when viewed on agarose gels, has often been regarded as a biochemical hallmark of apoptosis. As shown in Fig. 3A, DNA extracted from RINm5F cells, which had been exposed to human amylin for varying amounts of time, exhibits a characteristic fragmentation ladder upon agarose gel electrophoresis. These distinct DNA ladders were observed as early as 4 h following human amylin treatment of cells at passage 26. However, rat amylin did not induce apoptosis during equivalent exposures of up to 24 h, indicating again that sequence differences between human and rat amylin and/or its aggregation propensity are likely to be important factors in the induction of apoptosis. Equivalent results have also been obtained using a second pancreatic islet β-cell line, βTC6-F7 (J.-Z. Bai and G.J.S. Cooper, unpublished data). Our findings are consistent with results reported from studies using similar concentrations of human amylin (5–20 μM) in primary cultures of rat and human islet β-cells [10], indicating that the RINm5F cell line is a suitable cellular model for studying the molecular mechanism of human amylin-evoked apoptosis.

Apoptosis induced by human amylin was also studied in RINm5F cells at a higher passage number (passage 53). As shown in Fig. 3B, no distinct DNA ladders were observed until 16 h after human amylin treatment began. This suggests that the time course of induction of apoptosis is passage-dependent. RINm5F cells from passage 53 proliferated with a doubling time of about 36 h (data not shown) and thus have a lower rate of proliferation, which may be due to a longer cell cycle in comparison to cells from passage 26. Our observation that apoptosis is delayed in more slowly proliferating cells may suggest that susceptibility to human amylin-evoked apoptosis is related to the rate of progression through the

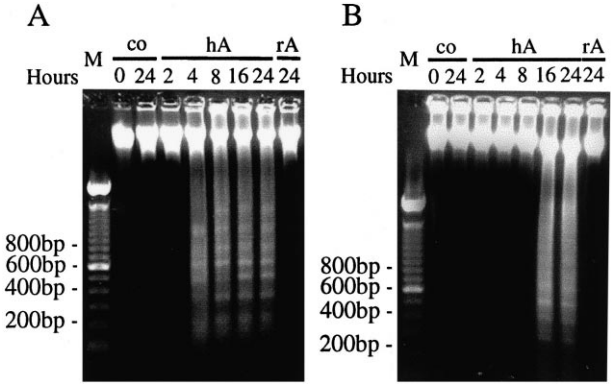

Fig. 3. Determination of DNA fragmentation. DNA was extracted from RINm5F cells from passage 26 (A) or passage 53 (B) at various time points after treatment with human amylin (hA), rat amylin (rA), or vehicle (water) control (co). M denotes the molecular size markers (100 bp DNA ladder).

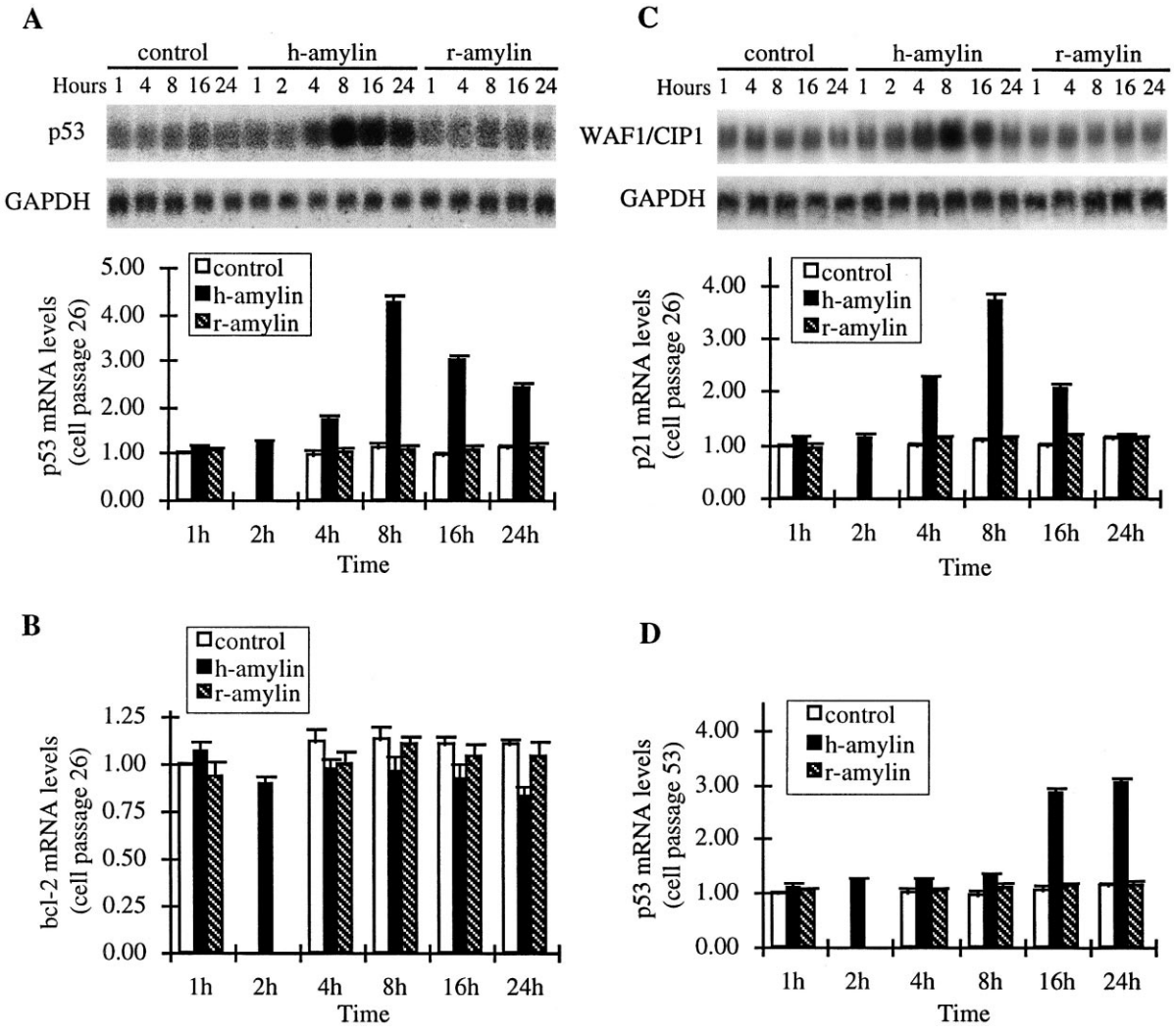

Fig. 4. Northern blot analysis for p53 (A,D), bcl-2 (B) and $p21^{WAF1/CIP1}$ (C) mRNA in RINm5F cells. Total RNA was isolated from RINm5F cells (passage 26 or 53) treated with human (h) or rat (r) amylin, or vehicle (water) control at the time points indicated. Northern blot patterns were reproducible in two further experiments. mRNA levels were quantified using a phosphor imager (BAS 2040). Results were normalized against GAPDH mRNA levels and calculated relative to control at 1 h. Each data point represents the mean ± S.E.M. of three determinations.

cell cycle. In addition, that cells which have a higher proliferative potential (passage 26) are more susceptible to apoptosis, may suggest that human amylin acts mainly on proliferating cells.

3.2. *Enhanced expression of p53 and $p21^{WAF1/CIP1}$ in RINm5F cells exposed to human amylin*

In patients with NIDDM, the number of β-cells decreases in association with increasing deposits of islet amyloid [39], which increase with worsening clinical severity [40]. Recent studies using an animal model of NIDDM (the Zucker diabetic fatty rat) have concluded that the decreased β-cell mass in NIDDM is likely to be caused by an increased rate of cell death by apoptosis, rather than a decline in the rate of β-cell proliferation [41]. Therefore, identification of genes implicated in human amylin-induced apoptosis could help to elucidate the cause and progression of the disorder. Human amylin may activate a subset of 'death genes' encoding specific molecular components of the apoptotic machinery in islet β-cells. Among the apoptosis-related genes, *p53* has been shown to act as a primary death-promoting gene, whereas *bcl-2* is a major apoptosis-suppressing gene; thus these genes could be involved in the process of human amylin-evoked apoptosis. The expression of p53 and bcl-2 in RINm5F cells treated with human amylin was therefore examined in this study by Northern blot analysis. The results revealed that levels of p53 mRNA are up-regulated, but that bcl-2 expression remains unchanged. As shown in Fig. 4A, the effect of human amylin on the accumulation of p53 mRNA appeared to be gradual and was not detectable within the first 3 h. An increase in p53 mRNA was, however, detected after 4 h, reaching maximum levels by 8 h. Thereafter the level of p53 mRNA remained relatively high compared with controls, although a slight decrease had occurred by 24 h. Relative mRNA levels of p53 were calculated by normalizing to levels of GAPDH mRNA, and by comparing amounts of p53 mRNA in amylin-treated cells with those in corresponding untreated cells. In contrast, bcl-2 mRNA was not increased over the period of 24 h (Fig. 4B). Rat amylin, by contrast, had no effect on the expression of either p53 or bcl-2. Our data indicate a role for p53 in human amylin-evoked apoptosis in cultured RINm5F cells. However, no significant change in bcl-2 expression was de-

tected, suggesting that the contribution of bcl-2 to the induction of RINm5F cell apoptosis is insignificant. The induction of p53 mRNA in response to human amylin treatment coincided with the initiation of apoptosis, as the DNA laddering pattern became detectable at the same time point. It was also found that the level of p53 mRNA in cells from passage 53 was not significantly increased until 16 h after human amylin treatment (Fig. 4D), suggesting a passage- and cell cycle stage-specific role for p53 in RINm5F cell apoptosis. Thus, it is likely that cells of the same lineage at different stages of maturation are likely to have different degrees of human amylin responsiveness.

p53 is one of the most frequently studied tumor-suppressing genes. Any reagent or genotoxic stress that produces DNA damage can cause a specific cellular response whereby the p53 protein accumulates to high levels [28]. This increase in expression of p53 seems to be responsible for the arrest of cells in the G1 phase of the cell cycle, presumably to enable cells to repair their damaged DNA prior to the onset of DNA synthesis and mitosis [29]. However, in the case of severe DNA damage, p53 may promote apoptosis to eliminate cells with compromised DNA. It has become increasingly evident that cells must progress to late G1 or early S phase in the cell cycle for apoptosis to occur [42]. This particular time in the cell cycle is known as the p53 restriction point. To determine whether human amylin-induced apoptosis in RINm5F cells is preceded by a growth arrest, cell cycle analysis was performed using a FACScan flow cytometer. Cell cycle distribution was determined by nuclear DNA content, as assayed by propidium iodide staining. The results indeed revealed a reduction of cells in the S/G2 phase 24 h after human amylin treatment (data not shown), suggesting that entry of cells into the S phase is hindered, thus provoking arrest in G1 of the cell cycle.

The mechanism by which p53 elicits apoptosis is still unresolved. p53 functions as a transcription factor and is able to bind in a sequence-specific manner to DNA, activating the transcription of several other target genes [28]. One of the genes for which transcription is induced by p53 is the CDK-inhibitor p21. p21 has been implicated in the induction of p53-dependent or -independent apoptosis in various cell types in response to a variety of agents [26,28] while other studies have also suggested that p21 may protect from apoptosis or may not even play a role in this process [43,44]. Therefore, the role of p21 in DNA repair and apoptosis remains controversial. p21 may exert an indirect influence to either induce or protect from apoptosis. In this study, the expression of p21 mRNA in human amylin-treated RINm5F cells was examined using Northern blots. The results, shown in Fig. 4C, demonstrate a significant increase of p21 mRNA, detected 4 h after human amylin treatment, which reached a maximum level at 8 h and was followed by a decrease at 16 and 24 h. In contrast, no changes in p21 mRNA expression were observed up to 24 h after rat amylin treatment. Our data suggest that enhanced expression of p53 in response to human amylin treatment may be followed by an increase of p21, resulting in decreased cell proliferation rates. This decreased proliferation rate was due to G1 arrest, as demonstrated by cell cycle analysis and occurrence of apoptosis.

It has been suggested that p53-mediated apoptosis is associated with abrogation of growth arrest and continued progression through the cell cycle [42]. Our experiments show that the level of p21 mRNA was increased at the same time point as p53 mRNA and subsequently decreased to the untreated control levels by 24 h, whereas p53 mRNA levels continued to remain relatively high (Fig. 4A,C,D). This finding implies that RINm5F cells may be arrested prior to apoptosis. It is possible that the increases in p53 and p21 mRNA in RINm5F cells occurring in response to human amylin initiate growth arrest and give sufficient time for DNA repair. The following decline in p21 mRNA may fail to elicit the response of G1 arrest, thus allowing continued progression through the cell cycle, leading to deficient DNA repair and subsequent apoptosis.

Apoptosis plays an important role in normal cell homeostasis and in the pathogenesis of a variety of human diseases [45,46]. Amyloid deposits are associated with progressive failure of cellular function in many forms of amyloidosis other than diabetes [45,47]. Neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease, have been characterized by increased levels of apoptosis resulting in excessive neuronal death [45]. Human amylin can assemble into fibrillar structures similar to those of Aβ peptide (amyloid β peptide), which accumulates in plaques in the brain of patients with AD. These two peptides with different primary but similar secondary structure are shown to be toxic to both islet β-cells and cortical neurons [10,47]. Immunoblotting and immunocytochemical studies also show that the level of p53 protein was significantly increased in glial cells of AD brains as compared to normal control brains [48]. The involvement of p53 in the apoptosis of islet RINm5F cells suggested that the mechanism of cell death induced by human amylin in islet β-cells may be similar to that evoked by Aβ peptide in AD brain.

In summary, our data demonstrate that exogenously applied human amylin can inhibit RINm5F cell proliferation by inducing growth arrest and apoptosis in a manner dependent upon passage number. Activation of this apoptosis pathway is associated with increased expression of p53 and p21. Further studies will be required to clarify whether p53 is directly associated with processes involving its site-specific DNA binding capacity, or whether it is indirectly increased by other factors induced by human amylin. Possible candidates could include the nuclear factors known to activate transcription of p53. In addition, the aqueous human amylin used for the cell treatments in these studies contains a mixture of the soluble amylin precursor, small protofibrils, and higher order fibrillar structures [19,38]. Human amylin toxicity appears to vary with aggregation state and is correlated with the acquisition of β pleated-sheet structure, which in turn promotes human amylin self-assembly and aggregation in solution [5,18,37]. Therefore, it will be of interest to further determine which of these states of human amylin elicits β-cell death.

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