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Short Communication

Human Islet Amyloid Polypeptide Expression in COS-1 Cells

A Model of Intracellular Amyloidogenesis

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Non-insulin-dependent diabetes mellitus is characterized by concurrent loss of β-cells and deposition of islet amyloid derived from islet amyloid polypeptide (IAPP). We have previously demonstrated that IAPP-derived amyloid forms intracellularly in humans with chronic excess insulin expression (eg, insulinoma and insulin receptor antibody-induced insulin resistance). To determine whether overexpression of IAPP results in intracellular amyloid in mammalian cells, we transfected COS cells with vectors expressing amyloidogenic human IAPP or non-amyloidogenic rat IAPP. Transfected COS-1 cells secreted comparable amounts of human IAPP and rat IAPP (2.1 to 2.8 nmol/L/48 hours). After 96 hours, 90% of cells expressing human IAPP contained amyloid fibrils and were degenerating or dead, whereas cells transfected with rat IAPP lacked amyloid and were viable. Thus, overexpression of human IAPP can result in intracellular amyloid formation that is associated with cell death, suggesting that intracellular amyloid may play a role in β-cell loss in non-insulin-dependent diabetes mellitus. (Am J Pathol 1995, 147:609–616)

Studies in macaques[1] and humans[2,3] suggest that loss of β-cell mass and accumulation of islet amyloid are related processes with islet amyloid replacing islet volume previously occupied by β-cells. Islet amyloid results from polymerization of islet amyloid polypeptide (IAPP, amylin),[4] a 37-amino-acid polypeptide derived from a larger precursor. IAPP is a normal product of β-cells[4] and is co-packaged and co-secreted in a soluble form with insulin[5]; its function has not been established.[6] In humans and cats with non-insulin-dependent diabetes (NIDDM), the molecular form of IAPP present in amyloid deposits is identical with the secreted form.[4,6,7] *In vitro* studies indicate that residues 20 to 29 of human, cat, and macaque IAPP is highly amyloidogenic, whereas in rats and mice, which do not develop NIDDM or islet amyloid, the homologous region is divergent and is not fibrillogenic.[8]

As hIAPP spontaneously aggregates into amyloid fibrils *in vitro*,[8] mechanisms presumably exist to traffic amyloidogenic human (h)IAPP through the secretory pathway in a soluble form. We have proposed that intracellular IAPP amyloidogenesis may occur if the normal trafficking mechanisms are impaired or if the IAPP synthetic rate exceeds the capacity of the trafficking mechanisms.[9,10] We also proposed that intracellular amyloid may be cytotoxic,[9,10] and recent studies have demonstrated that supraphysiological extracellular concentrations of IAPP or IAPP-

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derived amyloid cause cell death.[11,12] To test the hypothesis that overexpression of human but not rat (r)IAPP results in intracellular amyloidogenesis, we utilized a COS cell model in which hIAPP *versus* rIAPP was synthesized at a high rate (COS cells have previously been shown to be an effective cell type to examine protein synthesis and secretion of secretary proteins directed by transfected genes[13,14]). Moreover, this model system was effective in producing amyloid-like fibrils in transient transfection studies of the β/A4 domain of the Alzheimer amyloid protein precursor.[15]

Research Design and Methods

Plasmids

The cDNA (1000 bp) of hIAPP cloned into the *Eco*RI site of pUC18 was provided by Dr. Christer Betsholtz, University of Uppsala, Uppsala, Sweden, and the rIAPP cDNA (441 bp) cloned into the *Hinc*II site of pGEM4Z was obtained from Dr. Donald Steiner, University of Chicago, Chicago, IL. Both IAPP cDNAs contained the entire coding region for human and rat prepro-IAPP. The expression vectors pMT2, a derivative of p91023(B)[16] and pED4neo[17] were generously provided by Dr. Randall J. Kaufman, Genetics Institute, Cambridge, MA. Expression from pMT2 and pED4neo is under the control of the adenovirus major late promoter driven by the SV40 enhancer and both vectors incorporate the adenovirus tripartite leader sequence upstream of unique cloning sites to improve translational expression efficiency.[16,17] Amplification of the vectors can be achieved by treatment of cells with methotrexate because of the presence of the dihyrofolate reductase gene, which lies downstream of the unique cloning sites and which is co-expressed; however, this option was not employed in these studies. The pED4neo vector also contains a neomycin resistance gene just upstream of the dihydrofolate reductase gene to enable selection of permanently transformed cell lines. The hIAPP cDNA was excised by *Eco*RI digestion and cloned into pMT2[17] at the unique *Eco*RI site to form pMT2-hIAPP. The pMT2-rIAPP gene was made by removing the rIAPP cDNA insert by *Pst*I/*Eco*RI digestion and ligation into *Pst*I/*Eco*RI-digested pMT2. The hIAPP *Eco*RI fragment was inserted into the unique *Eco*RI cloning site of the pED4neo vector to generate hIAPP-pED4neo. To allow the insertion of the rIAPP cDNA into the dicistronic pED4neo vector, the *Pst*I/*Eco*RI rIAPP fragment was blunt ended by treatment with Klenow, *Eco*RI linkers were added, and the fragment was ligated into *Eco*RI-digested pED4neo.

Cell Transfections

COS-1 cells were purchased from American Type Culture Collection (Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Guithersburg, MD) supplemented with 10% Fetalclone (Hyclone, Logan, UT), 100 U/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), and 2 mmol/L L-glutamine (GIBCO BRL). Cells were maintained at 37°C in an atmosphere containing 5% CO_2 and 100% humidity. Confluent cells were rinsed with 10 ml of phosphate-buffered saline (PBS, GIBCO BRL) and harvested by digestion with 5 ml of trypsin (1X, GIBCO BRL) for 5 minutes. Cells were collected by centrifugation (5000 rpm for 1 minute), washed twice with 10 ml of media containing serum and resuspended in PBS containing 0.1% glucose at a concentration of 6.25×10^7 cells/ml. For each transfection, 4×10^7 cells (400 μl) were mixed with 15 μg of plasmid DNA (double $CsCl_2$ gradient purified) in 50 μl of 10 mmol/L Tris-HCl (pH 7.5) and 1 mmol/L EDTA, and the cells were electroporated (500 μF at 350 V; Gene Pulser, BioRad Laboratories, Richmond, CA) in standard cuvettes with a 0.4-cm electrode gap (BioRad). The electroporated cells were allowed to stand on ice for 7 minutes, resuspended in 0.8 ml of medium, and plated on 10-cm tissue culture dishes containing 10 ml of medium.

Cell Analysis

Cells were harvested at 48 and 96 hours by adding 2 ml of trypsin (1×). The cells were washed with 3 ml of medium containing serum, resuspended in PBS, fixed in 10% neutral buffered formalin solution for 30 minutes, washed in PBS with 0.1% Triton X-100 (PBS/T), and then incubated in 10% normal goat serum. After a PBS/T wash, cells were incubated overnight at 4°C with rabbit anti-hIAPP (1:1000, Peninsula Laboratories, Belmont, CA), washed with PBS/T, and incubated with goat anti-rabbit IgG-fluorescein isothiocyanate (4 hours at 23°C), then washed and resuspended in PBS/T. Immunofluorescence was evaluated with a Zeiss confocal microscope (He/Ar laser, $\lambda_{excitation}$ = 488 nm). For ultrastructural studies,[9] cells were harvested at 48 and 96 hours after transfection. Cell pellets were fixed with Trump's solution for 3 hours at 4°C, followed by overlaying the pellets with 0.1 mol/L phosphate buffer. The pellet was then placed in gelatin, dehydrated in acetone, and embedded in Spur's resin. Ultrathin sections were placed on copper grids and routinely stained with lead citrate and uranyl acetate. Additional sections were labeled for IAPP by the

immunogold technique. Briefly, thin sections were placed on celloidin membranes, etched with saturated sodium metaperiodate for 16 minutes, washed with water, and then incubated in Tris-buffered saline with 0.05% Tween (TBS-T) and 1% bovine serum albumin. Sections were then incubated with the primary antiserum (1:1000 anti-hIAPP1–37; Peninsula Laboratories) for 2 hours at 37°C and then rinsed with TBS-T. Sections were incubated with goat anti-rabbit immunoglobulin conjugated to 15-nm gold for 2 to 3 hours at room temperature and were then rinsed in TBS-T. The sections were counterstained with uranyl acetate. Immunocytochemical negative controls consisted of omission of the primary antiserum and application of the gold-conjugated secondary antibody solution alone. Sections were examined on a Jeol 1200 electron microscope. At 96 hours, 20 cells, each having specific rat and human IAPP-immunogold labeling, were identified by electron microscopy and evaluated for evidence of amyloid fibril formation, location of IAPP-immunogold labeling, and evidence of cellular degeneration.

The 48- and 96-hour conditioned media from COS cells transfected with pMT2-hIAPP and pMT2-rIAPP were frozen immediately after collection and stored at −80°C until assayed. IAPP concentrations were measured by radioimmunoassay as previously described[18] to determine (1) that COS cells are capable of secreting rIAPP and hIAPP and (2) to ensure that the extracellular concentrations of IAPP were less than the cytotoxic concentrations recently reported.[11,12] To assess IAPP toxicity in COS cells, synthetic hIAPP (Merrifield solid phase synthesis, Protein Synthesis Core, Mayo Clinic) was dissolved in 30% acetic acid (256 μmol/L) and diluted into COS cell media at final concentrations of 0.0256 to 256 nmol/L.

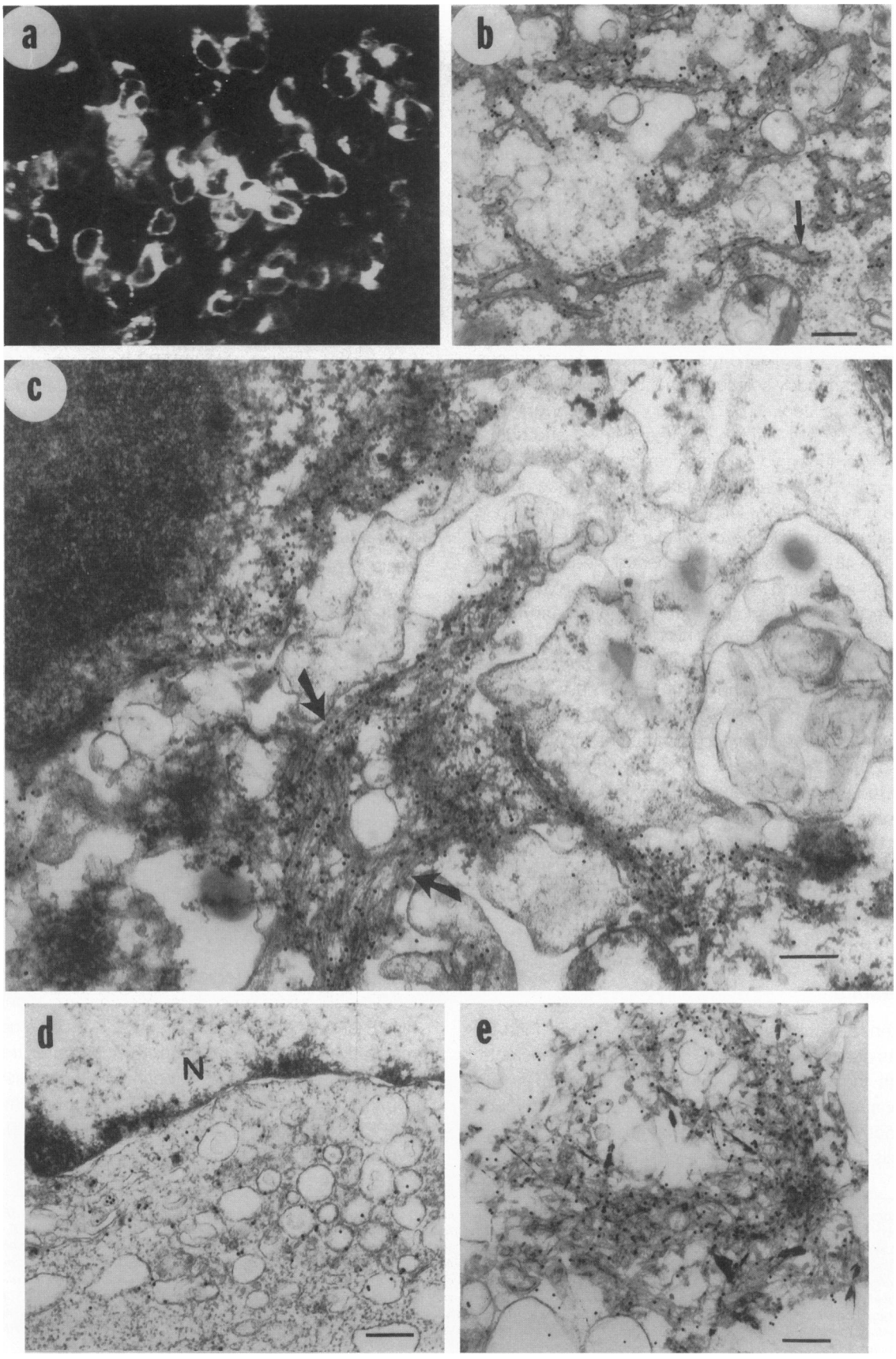
Results

Intense cytoplasmic immunofluorescence for IAPP was attained in 50 to 75% of COS-1 cells transfected with the pMT2-hIAPP (Figure 1a) and pMT2-rIAPP constructs (not shown). At 48 hours, hIAPP immunoreactivity was detected by immunogold labeling in cells transfected with the pMT2-hIAPP and pMT2-rIAPP genes in well demarcated regions of the cytoplasm represented by concentrated aggregates of tortuous endoplasmic reticulum-like structures (Figure 1b), in smaller vesicles, or apparently free in the cytoplasm. These cells were viable as assessed by the presence of intact plasma membranes, a normal complement of intracellular organelles, and a normal chromatin pattern.

At 96 hours, all of the cells (20 of 20) from the pMT2-rIAPP transfected group had IAPP-immunogold labeling patterns indistinguishable from the 48-hour patterns, lacked detectable amyloid fibrils, and had morphology consistent with viable cells (Figure 1d). In contrast, at 96 hours, 18 of 20 (90%) cells transfected with the pMT2-hIAPP gene exhibited intense IAPP-immunogold labeling in association with either loosely arranged fibrils or densely packed arrays of fibrils within the cytoplasm (Figure 1c). The fibrils (7 to 10 nm in diameter with indefinite lengths) closely resembled amyloid deposits observed in fixed pancreas tissue. All 18 cells exhibiting intracellular amyloid deposits appeared degenerate on the basis of extremely condensed nuclear chromatin, overall loss of cytoarchitectural detail, and disruption of plasma membranes. The two cells lacking amyloid deposits were viable and had labeling principally in endoplasmic reticulum-like structures. Cells transfected with pED4neo-hIAPP also showed intracytoplasmic amyloid fibrils and cellular degeneration at 96 hours (Figure 1e). It has recently been suggested that in Alzheimer's disease, β-protein aggregates intracellularl Ig as cytoskeletal-associated amyloid deposits.[15] In the present studies, we were unable to identify any fibrils in association with cytoskeletal filaments. In fact, in the hIAPP transfected COS cells, intracellular aggregates of IAPP were frequently identified adjacent to but distinct from cytoskeletal filaments (Figure 2).

As determined by radioimmunoassay, transfected COS-1 cells secreted comparable concentrations of hIAPP and rIAPP (2.8 ± 0.4 and 2.1 ± 0.2 nmol/L, respectively) into the medium 48 hours after transfection. In dose-response experiments to assess the cytotoxic effects of hIAPP on COS cells, 256 nmol/L hIAPP reduced cell growth by 21 ± 4% ($P < 0.01$) over a 48-hour time period but was without effect on

Figure 1. a: *Photomicrograph demonstrating strong immunofluorescence for IAPP in COS-1 cells 48 hours after transfection with pMT2-hIAPP.* b: *Electron micrograph demonstrating endoplasmic reticulum-like structures in a COS-1 cell 48 hours after transfection with pMT2-hIAPP. Note extensive IAPP immunolabeling (15-nm gold) within the endoplasmic reticulum-like structures (arrow). Bar, 250 nm.* c: *Electron micrograph of a degenerating COS-1 cell 96 hours after transfection with pMT2-hIAPP. Note the numerous, IAPP-immunolabeled (15-nm gold), 8- to 10-nm diameter, nonbranching fibrils (consistent with amyloid fibrils) free within the cytoplasm (arrows) and the marked condensation of chromatin in the nucleus (upper left) of this degenerating cell. Bar, 250 nm.* d: *Electron micrograph of a COS-1 cell 96 hours after transfection with pMT2-rIAPP. Note the normal chromatin pattern in the nucleus (N) and IAPP immunogold-labeled endoplasmic reticulum-like structures and vesicles in the cytoplasm. No amyloid formation is evident. Bar, 250 nm.* e: *Electron micrograph demonstrating amyloid fibrils with extensive IAPP-immunogold label in the cytoplasm of a COS-1 cell 96 hours after transfection with pED4neo-hIAPP. Bar, 250 nm.*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cell growth at lower concentrations. Thus, the cytotoxic effects of IAPP on COS cells was far higher than the concentrations obtained in the media in the current transfection studies.

Discussion

In the present studies we have demonstrated that high level expression of hIAPP (but not rIAPP) results in intracellular IAPP-derived amyloid formation. These results support our hypothesis that sufficiently high expression of amyloidogenic hIAPP in mammalian cells can result in intracellular amyloid formation. Although the mechanism is unknown, we have previously hypothesized that this may occur by overwhelming the normal protein-trafficking mechanisms thereby allowing excess IAPP to aggregate.[9,10] This transfected COS cell model provides a unique system in which to study the mechanisms of intracellular amyloidogenesis.

Intracellular IAPP-derived amyloid formation is of significant pathogenetic relevance to NIDDM in that IAPP-derived islet amyloid occurs in most, if not all, individuals with NIDDM and is associated with the concurrent loss of β-cell mass.[6] Furthermore, it was recently shown that hIAPP-derived amyloid fibrils induce islet cell death *in vitro*,[12] supporting a direct role for islet amyloid in the progressive loss of β-cells in NIDDM. In human insulinomas, intracellular deposits appear to precede extracellular deposits and therefore likely represent the initial site of amyloid formation.[9] Also, evidence for intracellular amyloid formation in cat[15] and macaque[19] models of NIDDM support the hypothesis that IAPP-derived islet amyloid present in patients with NIDDM forms intracellularly. Intracellular amyloidogenesis is not unique to the islet. Thus, intracellular amyloid has been described in myeloma,[20,21] pituitary tumors,[22] amyloid-producing squamous cell carcinoma of the cervix,[23] classical Alzheimer's plaques,[24] and inclusion body myositis.[25]

The molecular basis of amyloidosis *in vivo* is still poorly understood. First, it is clear that a potentially amyloidogenic protein is required, as illustrated by the present study in which hIAPP, but not rIAPP, resulted in amyloidogenesis, mimicking the *in vivo* state in which islet amyloid is present in humans (with NIDDM) but has not been described in rats. However, although many proteins are potentially amyloidogenic, in health, amyloid deposits are not formed. The latter implies that mechanisms must normally act to prevent amyloidogenesis and that these must be overcome in disease states characterized by amyloid formation. In some amyloids, there is an amino acid substitution in the responsible protein (eg, β-protein in hereditary Dutch type Alzheimer's disease,[26] transthyretin in senile cardiac amyloidosis[27] and gelsolin in Finnish hereditary amyloidosis[28,29]). The amino acid substitution in these proteins presumably causes either an increase in amyloidogenicity of the protein or a failure of appropriate chaperone protein recognition, binding, and therefore protection from aggregation. This situation is analogous to the failure of appropriate trafficking of the cystic fibrosis chloride channel protein resulting from the single amino acid substitution at site 509.[30] Other amyloids appear to develop as a result of excessive synthesis rates of the responsible protein (which has a normal amino acid sequence), eg, IAPP in insulinoma,[2] β-protein in Trisomy 21,[31] and immunoglobulin light chains in myeloma.[20,21] Of interest is that, although the overproduction of IAPP in neoplastic islet tissue in adults results in IAPP-derived amyloid,[19] we have recently shown that islet neoplastic tissue present in children with infantile hypoglycemia is not characterized by IAPP-derived amyloid.[32] Since the availability of chaperone proteins may be decreased with aging,[33,34] it is possible that the apparently increased vulnerability of aging adults to some local amyloids (eg, islet amyloid in NIDDM and cortical amyloid deposits in Alzheimer's disease) may reflect decreasing availability of chaperone protein-binding capacity.

Our observation of cell death associated with intracellular IAPP-derived amyloid fibril formation is consistent with a recent *in vitro* study that showed direct cytotoxicity of hIAPP-derived amyloid fibrils for human and rat islet cells, COS cells, PC12 pheochromocytoma cells, and primary aortic endothelial cells.[12] Also, hIAPP (like β-amyloid of Alzheimer's disease) but not rIAPP is toxic to rat primary hippocampal cultures.[11] It is noteworthy that in the latter two studies high concentrations of hIAPP were required for 50% killing of rat hippocampal neurons (20 μmol/L)[11] or rat and human islets (7 to 10 μmol/L).[12] As the levels of IAPP in our transfected COS-1 cell medium never exceeded 2.8 nmol/L, the transfected cells were being killed at hIAPP concentrations several thousandfold lower than those required to kill islet cells or hippocampal neurons.[11,12] Thus, it is unlikely that in our experiments COS-1 cells were killed by extracellular IAPP-derived amyloid. This suggests

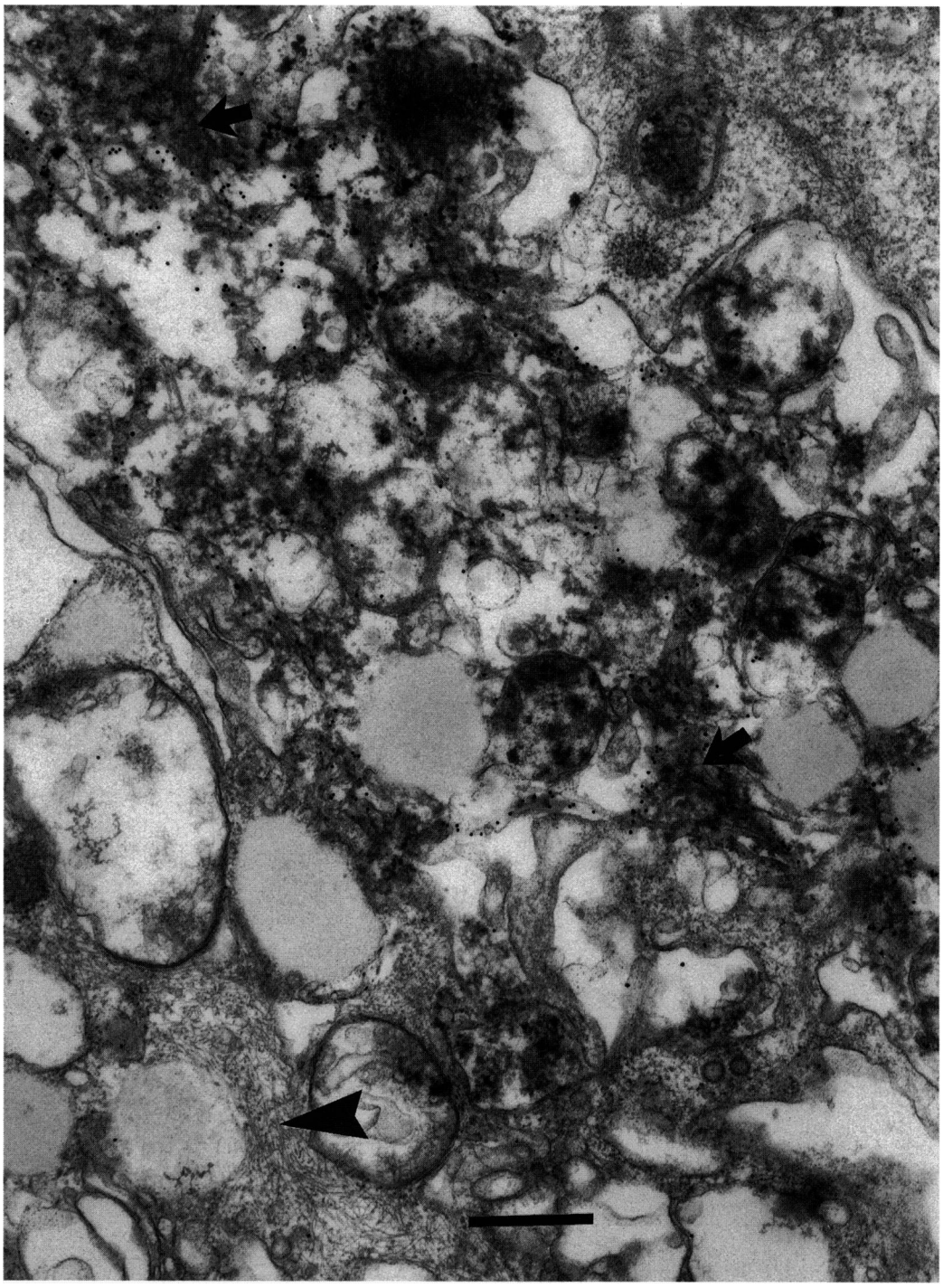

Figure 2. *Electron micrograph of a COS-1 cell 96 hours after transfection with pMT2-hIAPP. Early amyloid formation labeled intensely for IAPP by immunogold is evident. Note the adjacent cytoskeletal filaments (arrowhead) that are distinct from the IAPP aggregates (arrows) and have no IAPP labeling. Bar, 500 nm.*

that cells may be more sensitive to intracellular than extracellular deposition of hIAPP-derived amyloid.

To our knowledge, our data represent the first demonstration of an association of intracellular amyloidogenesis with cell death. Together these studies suggest that intracellular amyloidosis represents an underlying mechanism of cell death in islet cells in NIDDM as well as in other forms of amyloidosis. The mechanism of amyloid-induced cell death is not

known; however, it is possible that amyloid formation within the secretory pathway may disrupt this essential cellular transport function.

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