Insulin as an amyloid-fibril protein at sites of repeated insulin injections in a diabetic patient

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Summary. A patient with Type 1 (insulin-dependent) diabetes mellitus developed localised amyloidosis at the sites of his injections of porcine insulin. A major amyloid fibril protein was extracted and, by means of its amino acid composition and amino acid sequence, it was shown to contain intact insulin molecules. Porcine insulin is the tenth protein and the

first foreign protein to be chemically identified in human amyloid fibrils.

Key words: Diabetes mellitus, insulin, localised amyloidosis, amino acid sequence analysis.

Amyloid deposits have varied origins and chemical compositions, but they all give the pathognomonic green birefringence in polarised light when stained with Congo red, and possess a fibrillar ultrastructure. The common systemic forms are related to serum amyloid A (AA) protein or immunoglobulin light chains, but in localised amyloidosis the fibril proteins may contain hormone-like polypeptides – a procalcitonin in medullary carcinoma of the thyroid [1], and islet amyloid polypeptide (IAPP) in the pancreatic islets in Type 2 (non-insulin-dependent) diabetes mellitus [2–4] and in an insulinoma [2]. Insulin, also a polypeptide, has long been known to be capable of conversion in vitro to a fibrillary, amyloid-like, form [5–7]. Fibrillary insulin has recently been demonstrated in microscopic quantities around the needle tip after prolonged subcutaneous infusions of insulin in rats, and in a human diabetic subject [8]; clinically significant localised amyloidosis due to insulin has not, however, been reported hitherto. We describe amyloid deposits occurring in an insulin-dependent diabetic patient, and give the analytical findings of an amyloid protein that was extracted.

Case report

A young man of Afro-Caribbean ancestry, born in 1963, presented with weight loss, hyperglycaemia and ketosis in 1977. Type 1 diabetes mellitus was diagnosed and treatment instituted with porcine neutral soluble insulin ("Actrapid" insulin, Novo Laboratories Limited, Basingstoke, Hants, UK), twice daily, in doses initially totalling 36 units. He habitually gave his injections at the same two places, one in each thigh. Compliance was poor, and from 1981 he

ceased to attend the Diabetic Clinic, although he continued to obtain supplies of insulin through his General Practitioner, and he changed from using glass syringes, cleaned with alcohol, to disposable plastic syringes (Becton Dickinson UK Limited, Cowley, Oxford, UK). In June 1985, an abscess developed at the injection site in the left thigh and, after antibiotic therapy had proved unsuccessful, he was admitted to hospital. The swelling was drained surgically and a mass of firm tissue excised, and thereafter healing occurred satisfactorily. His insulin dosage, self-regulated, was now 176 units per day, but it is not known for how long it had been at this level; treatment was changed to a mixture of porcine neutral soluble and isophane insulins ("Mixtard 30/70" insulin, Nordisk-UK, Epsom, Surrey, UK), and the dose reduced considerably. The patient was advised to give his injections into the abdominal wall.

In August 1986, when his general health was good, investigations gave the following results: haemoglobin 15.5 g/dl, mean erythrocyte volume 88 fl, haemoglobin S absent, HbA_{1c} 12.4% (normal range 6–8%), and erythrocyte sedimentation rate 3 mm in 1 h. Normal results were obtained for serum electrolytes, urea, creatinine, calcium, phosphate, bilirubin, γ glutamyl transpeptidase, aspartate transaminase and alkaline phosphatase. The serum α -1 globulin was low but other fractions were normal, as were total protein and albumin; tests for antinuclear factor and for antibodies to smooth muscle, mitochondria and gastric parietal cells were negative. Chest radiography gave no abnormality. Evidence was found neither of systemic amyloidosis nor of anything that might have predisposed to it; proteinuria was absent and rectal biopsy was negative for amyloid.

'Lipohypertrophy' was noted at the injection site in the right thigh: this second lump was also excised. The patient could not remember when he had last given an injection at this site.

Methods

Histopathology

Tissues from the left thigh, fixed in formalin, and from the right thigh, fixed in phosphate-buffered formalin-glutaraldehyde, were embedded in paraffin wax, sectioned, and stained with haematoxylin-

Fig.1. Amyloid deposits (arrow) invested by multinucleated giant cells. Haematoxylin and eosin, $\times 260$

Fig.2. Electron micrograph showing typical fibrillar structure of amyloid (A); also cytoplasmic amyloid fibril inclusions (arrow) within a multinucleated giant cell. Osmicated, formalin-fixed specimen, uranyl acetate and lead citrate, $\times 27\,000$

in and eosin and Congo red. Cryostat sections of fixed tissue were stained with Oil red O; other frozen sections, and also small blocks of air-dried tissue, were mounted on graphite, placed in a JEOL 100C electron microscope equipped with a scanning electron microscopy (SEM) unit and a Link 860 energy-dispersive electron probe microanalyser, and qualitative elemental analysis carried out [9]. For transmission electron microscopy, tissue was post-fixed in osmium tetroxide and embedded in low viscosity epoxy resin; ultrathin sections were stained with uranyl acetate and lead citrate, and examined in an AEI Corinth 275 electron microscope.

The following procedures were performed on a fresh sample of the second skin mass, frozen soon after excision and sent later to Uppsala.

Immunohistochemistry

Staining was performed on formalin-fixed, paraffin-embedded material by the peroxidase-antiperoxidase method [10], with diaminobenzidine to visualise the reaction. Antibodies used included a monoclonal antibody to proinsulin and an antiserum to insulin (kindly donated by Dr. O. Madsen and Dr. L. Wide), and antisera to insulin B-chain, a B-chain rich fraction of insulin, and a synthetic undecapeptide of IAPP [2, 11], as well as to amyloid AA protein [12] and P-component [13]. Also used were antisera to the amyloid light chain (AL) proteins AL λ II, AL λ VI and AL κ III, obtained by immunising rabbits with purified AL proteins which had been typed by amino acid sequence analysis (unpublished data).

Purification and analysis of amyloid fibril protein

Approximately 2 g of frozen tissue was homogenised in 0.15 mol/l NaCl-0.05 mol/l sodium citrate-HCl buffer and centrifuged, and

this procedure repeated a number of times to remove soluble material (including any insulin that may have remained in its original form). After 3 further washes in distilled water, the remaining pellet was lyophilised. The dry material (about 50 mg) was defatted in methanol-chloroform (1:2), dissolved in 6 mol/l guanidine HCl-0.1 mol/l Tris HCl buffer, pH 8, containing 0.1 mol/l EDTA and 0.1 mol/l dithiothreitol, applied to a 1.6×60 cm Sepharose 6B-CL column, and equilibrated with 5 mol/l guanidine HCl in distilled water. Elution was performed with 5 mol/l guanidine HCl and monitored at 280 nm, and selected fractions were pooled, dialysed against deionised water and lyophilised. Material from the last retarded protein peak was dissolved in 1% sodium dodecylsulphate (SDS) in 0.05 mol/l sodium phosphate buffer, pH 7.2, and applied to a LKB HPLC apparatus equipped with a TSK-2000 column. Gel permeation HPLC was performed with 0.1% SDS in phosphate buffer and the eluted material recorded at 226 nm.

The amino acid composition of the protein was determined after acid hydrolysis. The N-terminal amino acid sequence was established using an automated gas phase sequencer and a phenylthiohydantoin amino acid analyser [3, 4].

Results

Pathology

The mass from the left thigh consisted of a skin ellipse with subcutaneous tissue 4 cm in diameter, containing extensive firm, tan-coloured areas; that from the right was similar but smaller. Paraffin sections confirmed

Table 1. Amino acid composition (non-oxidised sample) of the isolated purified major subunit protein of the skin amyloid fibrils, expressed as residues/molecule. Not determined = ND

	Insulin		Skin amyloid Fibril subunit
	Human	Porcine	
Asp	3	3	3.5
Thr	3	2	2.2
Ser	3	3	4.2
Glu	7	7	6.7
Pro	1	1	1.3
Gly	4	4	4.4
Ala	1	2	2.2
Cys	6	6	ND
Val	4	4	3.5
Ile	2	2	1.9
Leu	6	6	5.5
Tyr	4	4	3.1
Phe	3	3	2.3
His	2	2	1.7
Lys	1	1	1.4
Arg	1	1	1.3

Table 2. Comparison of the amyloid protein amino acid sequences with the known sequences of porcine insulin B- and A-chains. Cysteine (C) is not detected by the method used

Porcine insulin B-chain (upper), compared with the sequence found (lower)

	10	20	30
FVNQHLCGSHLVEALYLVCGERGFFYTPKA			
FVNQHL GSHLVEALYLV GER			

Porcine insulin A-chain (upper), compared with the sequence found (lower)

	10	20
GIVEQCCTSI C SLYQLENYCN		
GIVEQ TSI SLYQLENY		

Table 3. Amyloid fibril proteins identified by amino acid sequence

Precursor	Type of amyloid
1. Serum amyloid AA protein	Reactive systemic (secondary) amyloidosis (AA)
2. Immunoglobulin light chains	Monoclonal gammopathy- and myeloma-associated amyloidosis (AL)
3. Prealbumin	Familial and senile forms of systemic amyloidosis
4. β_2 -microglobulin	Amyloidosis associated with long term haemodialysis
5. Procalcitonin	Amyloid in medullary carcinoma of thyroid
6. Cystatin C	Icelandic familial cerebrovascular amyloidosis
7. β -protein	In Alzheimer's disease, Down's syndrome, senile dementia
8. Islet amyloid polypeptide (IAPP)	In islets of Langerhans in Type 2 diabetes
9. Atrial natriuretic peptide	Isolated atrial amyloid
10. Insulin	Iatrogenic amyloid (present case)

the presence of suppuration on the left; in addition, amorphous eosinophilic material was abundant in the dermis and subcutis of both specimens, arranged in irregular-shaped or nodular masses. This material was positive to the Congo red stain, and gave green birefringence when examined under polarised light. Pretreatment with potassium permanganate [14] failed to abolish the congophilia. Most of the deposits were sparsely cellular, but multinucleated giant cells were present (Fig. 1), occasionally numerous, and were closely applied to the amyloid material. Transmission electron microscopy revealed closely-packed microfibrils 9 nm diameter (Fig. 2), and cytoplasmic fibrillary inclusions were found within some of the giant cells (Fig. 2). Much collagen was also present in the stroma.

Frozen sections revealed small amounts of diffuse Oil red O-positive material in the areas that also contained amyloid, although the distribution was not identical. The oil could be removed by alcohol or other fat solvents, leaving the amyloid apparently unchanged. In addition, a few multinucleated giant cells contained minute lipid droplets. Using SEM, electron probe microanalysis of most areas revealed only the Bremsstrahlung, but a few particles gave the spectra of sulphur or calcium; a few others contained aluminium, gold, silicon, silver, titanium or zinc, but these were thought to be contaminants.

Immunohistochemistry

Strong binding was observed between the amyloid and the antibodies to insulin, insulin B-chain, the B-chain rich insulin fraction and amyloid P-component. Results with other antibodies were negative.

Amino acid analysis

The amino acid composition of the purified amyloid subunit protein proved to be similar to that of insulin, resembling the porcine hormone more closely than the human (Table 1). The high value for serine was probably false.

The amino acid sequence analysis yielded two amino acid derivatives at most steps, in equal amounts, corresponding to the two chains of insulin. Positions 1-19 of the A-chain and 1-22 of the B-chain were deduced; derivatives could not be identified at positions 6, 7 or 11 of the A-chain or 7 and 19 of the B-chain, suggesting the occurrence of cysteine at these points (the method used does not detect cysteine). In other respects virtual identity was established with the known sequence of the molecule of porcine insulin (Table 2).

Any residual free insulin there might have been at the injection site was considered to have been removed by the extraction procedure, nor was an alternative amyloid protein identified. The fibrils were therefore shown to contain complete, uncleaved, pig insulin molecules.

Discussion

The pathogenesis of amyloid formation in our patient remains to be elucidated. Most amyloids are believed to be formed by partial proteolysis of a larger precursor, which in the case of localised amyloidosis is expressed close to the site of deposition; the lower molecular weight fragments produced are thought to polymerise spontaneously into fibrils. In the present instance, proteolysis would appear to be excluded.

Fibril formation or aggregation of insulin can be induced in vitro in a number of ways, such as by acidity and heating and freezing, isoelectric precipitation, or mechanical agitation [5, 15–17], but there was nothing to suggest that any of these was involved. The silicone oil (dimethylpolysiloxane) used to lubricate plastic syringes is regarded as potentially harmful [18], since silicones may cause lipogranulomas and connective tissue diseases [19]; except for scanty particles, however, attributed to contamination, the tissues contained no demonstrable silicon. The giant cells were so closely related to the amyloid deposits that there is strong reason for believing that they were reacting to these rather than to other substances (less likely, they were actually forming the fibrils). A giant cell response to amyloid is an occasional finding in other circumstances [20]. Whether silicones facilitate insulin fibrillation is uncertain. The identity and significance of the oily material found remain obscure.

Nine amyloid proteins have been identified hitherto (Table 3). Insulin evidently shares with other polypeptides, including procalcitonin and IAPP, the ability to assume a fibrillary form in vivo. It is the tenth protein whose identity has been established by chemical analysis in human amyloid, the fourth related to a polypeptide hormone, and the first of iatrogenic origin. Amyloid P-component was also demonstrable in the deposits, as it has been in every other amyloid except that in the intracortical plaques of Alzheimer's disease.

Symptomatic localised amyloidosis at injection sites appears to be exceptional, but some of the lumps labelled 'lipohypertrophy' in diabetic patients who repeatedly use certain places they know to be pain-free might conceivably represent unrecognised amyloidosis. Also, in view of the report by Störkel et al. [8], subclinical amyloid formation after prolonged subcutaneous insulin infusions may be less rare than previously thought.

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