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Review article

Where disease pathogenesis meets protein formulation: Renal deposition of immunoglobulin aggregates

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Abstract

Aggregation is one of the important issues encountered during the development of immunoglobulin-based drugs. The aim of the current review is to discuss the causes and consequences of immunoglobulin aggregation as well as the relevance of immunoglobulin aggregation to disease pathogenesis. Extracellular deposition of immunoglobulins, either monoclonal light chains or intact polyclonal antibodies, induces renal failure in various nephropathies. The aggregates can present fibrillar or amorphous structures. In this review, factors known to influence protein aggregation, such as the primary structure of the protein, local environment and glycosylation are assessed, as well as the subsequent altered clearance, fibril formation and toxicity. The role of the protein local environment is emphasized. Even if the local environment causes only minor perturbations in the protein structure, these perturbations might be sufficient to trigger aggregate formation. This fact underlines the importance of choosing appropriate formulations for protein drugs. If the formulation provides a slightly destabilizing environment to the protein, the long-term stability of the drug may be compromised by aggregate formation.

Keywords: Immunoglobulin; Aggregation; Disease; Formulation; Therapeutic proteins; Amyloid

1. Introduction

There are many classes of protein where aggregation is associated with a disease. Over the last decades, much research has focused on proteins such as the amyloid beta protein and alpha synuclein. Less known is the fact that the aggregation of monoclonal or polyclonal immunoglobulins and subsequent renal damage take place in fatal diseases such as primary (AL) amyloidosis, light-chain deposition disease, heavy-chain deposition disease, light- and heavy-chain deposition disease, cast nephropathy and IgA nephropathy (Berger's disease) [1,2]. Although IgA nephropathy has a higher prevalence in Asian countries, representing 40–50% of all glomerulonephritis in Japan, Singapore and Hong Kong, IgA nephropathy has also become the most common form of glomerulonephritis in other industrialized countries [3,4].

Fig. 1 schematically presents the different diseases caused by immunoglobulin aggregation and Table 1 summarizes the relevant literature for each disease. In AL amyloidosis, monoclonal immunoglobulin light chains are deposited in the form of amyloid fibrils. In the case of light-chain, heavy-chain and light- and heavy-chain deposition diseases (LHCDD), which are different forms of the same pathology, monoclonal light or heavy chains are deposited in the form of amorphous aggregates [5]. These aggregates (fibrillar or amorphous) are found in the glomeruli and eventually cause renal failure. Their presence can also be detected in other tissues; cardiac deposits for instance, present in $>50\%$ cases of AL amyloidosis, are associated with shorter survival [6,7]. In cast nephropathy or 'myeloma kidney', filtered light chains bind to Tamm-Horsfall Protein (THP) in the thick ascending limb of the loop of Henle and form aggregates, obstructing the tubule fluid flow [8,9]. In IgA nephropathy, polyclonal intact IgA and complement component 3 (C3) deposit in the glomeruli, causing a glomerulonephropathy [2].

Immunoglobulins are present in relatively high concentrations in the blood stream, and even though they share most of their structure, their different aggregation patterns lead to

Abbreviations: C3, complement component 3; CDR, complementarity-determining region; COS-1, African green monkey kidney cells; DTT, dithiothreitol; FcαR, Fc receptor for IgA; IgA, immunoglobulin A; IgG, immunoglobulin G; IL, interleukin; LHCDD, light-chain, heavy-chain or light- and heavy-chain deposition disease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TGF, transforming growth factor; THP, Tamm-Horsfall protein; TNF, tumour necrosis factor.

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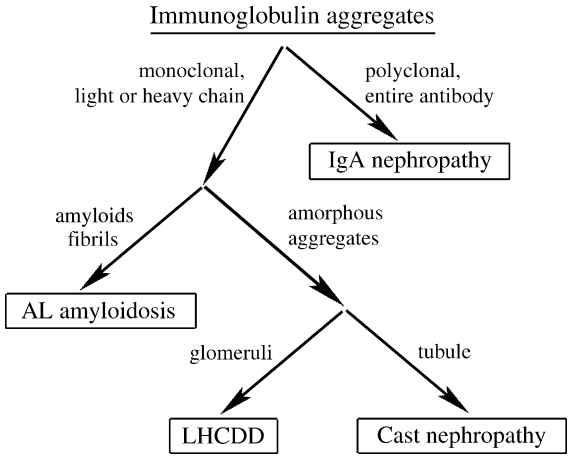

Fig. 1. Schematic description of the different diseases (in boxes) caused by immunoglobulin aggregation. The diseases are classified according to the following parameters: the nature of the protein, the type of aggregates and the localization of the renal damage.

different diseases. The fact that the aggregation of such endogenous proteins can cause a fatal disease highlights the importance of understanding the factors influencing immunoglobulin aggregation.

Immunoglobulins are also used as drugs and currently there are more than a dozen products on the market. Aggregation is one of the issues encountered during formulation that often compromises the stability of protein drugs. Little is known about the fate of the therapeutic immunoglobulins once injected into the human body and minimizing aggregation is one step towards reducing potential toxicity of protein drugs.

In this review, we discuss the potential causes of immunoglobulin aggregation in renal diseases and implications on the rational development of stable pharmaceutical formulations of proteins. Relevant factors influencing protein aggregation, such as the immunoglobulins' primary structures, their local environment and their glycosylation will be presented. The current knowledge regarding the mechanisms governing aggregate formation will be summarized. The toxicity of the aggregates to the kidney will also be described.

2. Causes of immunoglobulin aggregation

2.1. Influence of the primary structure

In AL amyloidosis, LHCDD and cast nephropathy, the immunoglobulin deposits are constituted of monoclonal light chains and light chain fragments. These light chains can either form fibrillar (amyloid) or amorphous aggregates, and present different tropisms. The fact that the immunoglobulins responsible for the disease come from a single clone has suggested that the amino acid sequence of the protein could explain the tendency to aggregate [1].

In order to describe the primary structure elements, which could cause these diseases, two factors were considered: the light chain subtype and the amino acid mutations. Immunoglobulin light chains are either from the κ or from the λ type. Each of these types is divided in subtypes: κ_I to κ_{IV} and λ_I to λ_{VIII} [10]. Serologic studies and sequence analysis of amyloid deposits extracted from more than 50 patients have shown that almost all subtypes can be involved in AL amyloidosis [1]. Another study has demonstrated that the λ_{II} subtype was overexpressed in patients suffering from AL amyloidosis, multiple myeloma or Waldenström's macroglobulinemia [11]. However, only the λ_{VI} subtype has been recognised as particularly amyloidogenic and is found exclusively in patients with AL amyloidosis [11].

As opposed to AL amyloidosis, κ light chains are involved in LHCDD twice as often as λ light chains, but less structural data are available and no particular subtype has been associated with the disease. In cast nephropathy, the tendency of light chains to aggregate seems to be independent of the light chain type [5]. This could be explained by the fact that in cast nephropathy, aggregates are formed through the interaction of light chains with Tamm-Horsfall Protein, and this interaction takes place in the complementarity-determining region (CDR) 3 [12]. As the domain responsible for aggregation is located in one of the hypervariable regions, the light chain subtype is less susceptible to being directly involved in aggregation.

Even though in AL amyloidosis, amyloid material contains fragments of the *constant* region [13–15]; the main constituent of the aggregates is the *variable* region of the light chain. The natural variability of this domain mitigates against the designation of a single amino acid or an amino acid sequence as responsible for aggregation. Furthermore, amyloid fibrils are cross-β-structures and light chains natively contain a considerable amount of β-structure. One situation where single amino acid substitutions could trigger aggregate formation would be by promoting the exposure of hydrophobic residues, destabilization of secondary structures, enhanced sensitivity to proteolysis or an increase in the population of intermediates in the folding–unfolding process. For example, Pro-40 is a highly conserved amino acid in immunoglobulin light chains [16] and a single mutation involving replacement with Leu could be responsible for fibril formation [17]. In this case,

Table 1
Classification of immunoglobulin-related diseases and corresponding literature

Disease	Precursor protein	References
AL amyloidosis	Monoclonal light chain, predominantly of the λ subtype	[1,6,7,11,13–16,18–23,25,28–30,32,35,36,42–45,47–52,56,62,68–70,72,73,76–78,81]
LHCDD[a]	Monoclonal light chain, predominantly of the κ subtype	[1,5,19,25–27,34,35,78]
Cast nephropathy	Monoclonal light chain	[8,9,12,54,70]
IgA nephropathy	Polyclonal entire IgA	[2–4,57–61,63–67,79,80,82–93]

[a] Light-chain, heavy-chain and light- and heavy-chain deposition diseases.

the aggregation behaviour of the benign immunoglobulin LEN was controlled by a single amino acid change. Interestingly, fibrillation of the benign immunoglobulin was also triggered by seeding with aggregated pathogenic fibrils, indicating that amino acid mutation is not the only mechanism responsible for the fibrillation phenomenon.

Sequence analysis of 180 human monoclonal light chains allowed the identification of other amino acid positions that can induce amyloid fibril formation. These positions are found in κ as well as in λ subtypes, and in the framework as well as in the CDR regions. They include residues 13, 20, 31, 32, 40, 45, 49, 50, 51, 52, 55, 60, 70, 74, 89, 90, 92, 93 and 96 [18]. However, the higher frequency of mutations of these residues in relation to amyloidosis has not allowed one or several of them to be assigned as being responsible for immunoglobulin fibrillation. Several other positions were shown to be altered in disease-related immunoglobulins, and mutations should be considered as modulators of immunoglobulin aggregation. Salt bridges mostly occur between framework regions of immunoglobulins and contribute to the stability of the protein [16]. For example, in light chains, Arg-61 and Asp-82 form a key, conserved salt bridge between two adjacent loops. Mutations on these residues lead to the destabilization of the protein. Replacing Arg-61 with a neutral residue like asparagine leads to amyloid fibril formation in vitro, whereas replacing Asp-82 with a neutral residue like isoleucine leads to amorphous aggregate formation [19]. The influence of others factors is underlined by the fact that the replacement of Asp-82 by the hydrophobic residue Leu is one of the features observed in a light chain that forms amyloid fibrils in vivo [20]. Many other residues may be significant in altering protein folding and stability, especially in positions 10, 65 and 106 [20], in positions 57, 61, 68, 78 and 84 [21] as well as in positions 25, 68 and 95 [22]. Unusual substitutions are also implicated in AL amyloidosis at positions 2, 29, 58, 72 and 85 [23]. All the substitutions can involve the replacement of a charged amino acid by a neutral one or the replacement of a neutral amino acid by a charged one. Destabilization of β-sheet edges could lead to interactions with other β-sheets and formation of amyloid cross-β-structures [24]. Evidence of destabilization of the sensitive β-sheet edges region was obtained from molecular dynamics studies [25].

In the case of LHCDD, amino acid substitutions introducing hydrophobic residues at the surface of the light chain are likely to play a role in aggregation. Interactions between hydrophobic residues of light chains might lead to aggregation and tissue deposition [26,27].

Two origins were proposed for the amino acid substitutions in immunoglobulins leading to aggregation diseases. One is the utilization of a particular germline gene, and the other is somatic mutations. There is evidence to support both hypotheses. A germline gene, called IGLV6S1, has been shown to encode for light chains of the λ_{IV} subtype, which is highly associated with amyloidosis [28]. This germline gene is also associated with the organ tropism: patients whose immunoglobulins were derived from the IGLV6S1 donor had dominant renal involvement and also presented cardiac and to a lesser extent nervous system involvement [7]. Two other gene segments have been demonstrated as being overexpressed in amyloidosis [29]. On the other hand, there is statistical evidence of antigenic selection in amyloidogenic light chains [30,31]. Somatic hypermutation and antigen selection might therefore modulate the influence of germline genes, either stabilizing or destabilizing the immunoglobulin fragments [30].

The focus on the amino sequence of the light chains should not overlook the importance of cysteinylation. Cysteinylation is the formation of an S-S bond between a cysteine residue of the protein and a free cysteine that can influence the immunoglobulin structure. Light and heavy chains are linked by interchain disulfide bonds. When free light chains are secreted in excess, cysteinylation between light chains is observed because no heavy chains are available for the correct disulfide bond formation. Analytical methods using reducing agents such as DTT or mercaptoethanol in the amino sequence determination process make the study of interchain cysteinylation impossible [32].

Based on current knowledge, monoclonal immunoglobulin deposition diseases can be considered as conformational diseases. However, primary structure alone does not allow differentiation between pathogenic and benign immunoglobulins [33,34]. Destabilizing mutations contribute to immunoglobulin aggregation and deposition, but nothing sequence-specific impedes benign light chains from forming fibrillar aggregates [35,36]. It has even been suggested that most monoclonal light chains could form amyloid fibrils [18]. Other factors that influence aggregation need to be considered, such as the local environment of the protein or post-translational modifications such as glycosylation.

This situation can be compared to the biotechnological engineering of therapeutic antibodies. The hybridoma technique allows the mass production of antibodies, after the immunization of a mouse with an antigen. Murine antibodies are immunogenic and there is a great interest in reducing the murine sequences in the final product to reduce immunogenicity. This process is called ‘humanization’. The successful total humanization of antibodies reduces immunogenicity, but cannot suppress it totally, showing that primary structure alone cannot address all issues regarding immunoglobulin stability or toxicity [37]. Modifications in the sequence of engineered therapeutic antibodies (e.g. during humanization or in order to increase activity) can potentially compromise the stability of the final product. The impact of sequence modifications on stability and potential aggregate formation is difficult to predict [16,38]. Research performed on disease-related immunoglobulins revealed at least four sites of potential destabilization. In immunoglobulins, highly conserved residues such as Pro-40 cannot be replaced without triggering protein aggregation [16,17]. The edges of naturally occurring β-sheets can potentially interact with any β-strand they encounter [24]. Thus, sequence modifications at the edges of β-sheets are prone to lead to aggregation [25]. Residue changes in the framework regions are more susceptible to compromise stability than changes in the CDR [16]. Swapping charged residues for hydrophobic ones (or the opposite) could

be potentially problematic, especially if it would lead to the exposure of hydrophobic residues or domains [26,27].

2.2. Influence of the local environment

Amyloid fibrils possess common structural features: they are straight and unbranched, mainly composed of cross-β-sheets, and possess the ability to bind dyes such as Congo red and Thioflavine T. At least 20 different proteins are known to adopt this class of fibril structure. This wide range of proteins able to form amyloid aggregates suggests that the phenomenon is a generic feature of polypeptide chains, and not specific to particular amino-acid sequences [39].

For example, acylphosphatase, a protein that is not related to any known amyloid disease, has been induced to form amyloid protofilaments and fibrils by incubation in a solution containing moderate concentrations of trifluoroethanol. Even though the particular conditions used in this experiment should not be directly correlated to in vivo fibril formation, they however suggest that amyloid formation is an intrinsic property of many proteins [40].

Amyloid aggregates formed by some non-disease-related proteins are inherently highly cytotoxic [41]. The aggregates formed by the SH3 domain of bovine phosphatidyl-inositol-3′-kinase (PI3-SH3) or the *N*-terminal domain of the *E. coli* HypF protein (HypF-N) have been shown to reduce the viability of mouse fibroblasts (NIH-3T3) and rat pheochromocytoma (PC12). This toxicity, evaluated by the MTT test, was only observed when the proteins were aggregated [41]. Again, the pathogenicity of protein aggregates would seem to be related to the structure of the aggregates and not to the sequence of the protein [41]. In some amyloid diseases, the mature fibrils are considered responsible for the toxicity, but in some cases the non-fibrillar, 'loose' aggregates may be the primary toxic species [41].

In the case of monoclonal deposition diseases, the conformation of an amyloidogenic light chain (SMA) has been studied during fibrillation [42], showing that the transition states for fibril formation were similar to the native state. Therefore, even if the local environment causes only minor perturbations in the protein structure, these perturbations might be sufficient to trigger aggregate formation [42]. This fact underlines the importance of choosing appropriate formulations for protein drugs. If the formulation provides a slightly destabilizing environment to the protein, the long-term stability of the drug may be compromised by aggregate formation.

The extracellular nature of protein deposits reinforces the hypothesis that aggregate formation can be triggered by environmental factors. When aggregated or misfolded proteins are produced by a cell, they are not released but degraded or concentrated in aggresomes. This behaviour has been observed for amyloidogenic SMA light chains expressed in COS-1 cells: the light chains were not released, but were found in perinuclear aggresomes [43]. If similar events take place in the human body, either the aggresomes will lead to cell death and the subsequent release of aggregated material, or aggregation will be triggered outside the cell by external factors [43].

A protein can be in contact with different surfaces, in the container used for storage and dispensing as well as in the blood circulation. The nature of the surface can trigger aggregation, as well as influence the type of aggregates: amorphous or fibrillar. In the case of AL as well as other amyloidoses, microdomains of membrane lipids known as 'lipid rafts' were proposed to allow amyloid formation [44]. When placing SMA light chains in contact with native mica, fibril formation rate is increased and formation of amorphous aggregates is inhibited [45]. The importance of the surface type was also underlined in in vitro fibril formation of immunoglobulin light chains, where the nature of the cuvette used in the experiments (quartz, polystyrene or polymethacrylate) is reported to influence the fibrillation phenomenon [46].

The pH micro-environment is another factor involved in immunoglobulin aggregation. The fibrillar or amorphous nature of the aggregates has often been related to the isoelectric point of the immunoglobulin. In the majority of cases, proteins isolated from patients with AL amyloidosis presented isoelectric points between 3.8 and 5.2, whereas proteins possessing basic isoelectric points have been associated with the amorphous deposits present in LHCDD. However, an immunoglobulin light chain extracted from the urine of an AL amyloidosis patient showed a highly basic isoelectric point. This protein forms fibrils in vitro exclusively at acidic pH values of 4.5 and 5.5 and only amorphous aggregates at physiological pH values of 6.5 and 7.4 [47]. Therefore, it was suggested that the tissue pH influences aggregate formation in this patient [47]. Similarly, the amyloidogenic GRY light chain has been shown to form amyloid fibrils in vitro in phosphate buffered saline at pH 7 but amorphous aggregates in acidic buffer at pH 3.5 [48].

Further evidence that environmental conditions govern protein aggregation is coming from studies of the amyloidogenic SMA light chain. Ionic strength and pH both influenced the type of SMA aggregation intermediates observed by atomic force microscopy [49]. The SMA light chain has been shown to aggregate upon exposure to copper ions, in cells as well as in vitro without the application of shear forces that are usually needed to form fibrils in vitro [50].

The variety of environmental variables influencing protein aggregation underlines the importance of taking into account the local environment of the protein. Thus, the composition of renal fluids might be a determinant factor. In the case of cast nephropathy, where filtered light chains form aggregates in the thick ascending limb of the loop of Henle, it has been shown in vitro that an increase in the concentration of sodium or calcium chloride enhanced the aggregation of cast-forming proteins. In addition, in vivo studies of a microperfused loop segment of rat nephron showed that the presence of the cast-forming proteins inhibited chloride absorption, resulting in an increased Cl^- concentration [8]. Consequently, the electrolyte composition of renal fluids appears to play an important role in cast nephropathy, especially because binding of light chains to THP and the subsequent cast formation take place in the tubule.

Methods to trigger in vitro fibril formation through agitation or heating as well as in situ monitoring with Thioflavine T have been reviewed [46]. These methods showed an increase in fibril formation when amyloidogenic or non-amyloidogenic light chains were incubated with renal solutes [51]. Mechanical stress caused by agitation probably triggered aggregation of both amyloidogenic and non-amyloidogenic light chains. It was discussed earlier that non-pathogenic light chains could aggregate under the appropriate conditions [40]. However, when samples are not submitted to mechanical stress, in the presence of simulated nephron solutes, 33 of 35 pathologic light chains can aggregate, whereas only one of five non-pathologic light chains formed aggregates [52]. These observations are the basis of a patent describing the diagnosis of diseases caused by protein aggregation [53].

Simulated renal conditions have also been used to develop treatments for cast nephropathy; in vitro as well as in vivo experiments demonstrated that an intraluminal presence of calcium ions or furosemide augmented tubular obstruction, whereas colchicine inhibits cast formation [9,54]. In vitro experiments showing reduced intraluminal aggregate formation may not predict the overall efficacy of a drug.

Similar parameters influence the aggregation of therapeutic immunoglobulins in the context of liquid formulation. Ensuring the stability of immunoglobulin formulations, as well as providing a practical way to administer them is not a trivial task. There is a need for stable concentrated solutions of immunoglobulins, allowing the administration of hundreds of milligrams per dose. Both chemical and physical stability (secondary structure and aggregation) have to be addressed when formulating a therapeutic immunoglobulin. Lyophilization is often used to ensure the chemical stability of the protein and to achieve high concentrations. However, reconstituted solutions are not exempt from aggregation problems and the instructions for use often state that the solution has to be reconstituted slowly and that shaking must be avoided. Thus, when designing liquid formulations of immunoglobulins, the influence of the type of buffer or of the ions present in the solution have to be taken into account to minimize the aggregation phenomena. The diversity of protein structures and properties has not yet allowed drawing general stabilization rules [38]. Rational choice of formulation should be based on experimental evidence obtained with the protein of interest. Sensitive methods need to be developed to assess protein aggregation in formulations at therapeutic doses as well as after injection into the body [55]. Work performed in our laboratory suggests that in vitro studies of therapeutic protein aggregates in biological fluids may be used in protein formulation selection in early development stages.

2.3. Influence of glycosylation

In AL amyloidosis, many light chains are glycosylated, usually in the variable domain. Glycolysation could induce a conformational change in the protein and trigger fibril formation [56]. This hypothesis can explain why amyloid fibrils extracted from the spleen of a patient contained glycosylated as well as non-glycosylated light chains [56]. The role of glycosylation is however better understood in IgA nephropathy, where the entire immunoglobulin is involved in the pathogenesis. IgAs possess *O*-linked oligosaccharides in the hinge region, unlike the majority of serum proteins, which are *N*-glycans. Even though the function of these glycans is not fully understood, they may play a role in stabilizing the three-dimensional structure of the protein. Unusual glycosylation in the hinge region results in the aggregation of the IgA molecule in IgA nephropathy [57]. This was shown in vitro by digesting serum IgA1 from healthy individuals with appropriate enzymes with a view to obtaining asialo, agalacto and 'naked' IgA1. The removal of the carbohydrates resulted in non-covalent self-aggregation of the immunoglobulins, as well as increased binding to extracellular matrix proteins. The protective role of the glycans and the idea that underglycosylation is involved in the pathogenesis of IgA nephropathy were consequently reinforced [58]. Furthermore, the presence of sialic acid has been identified as playing a role in inhibiting IgA1 self-aggregation [59]. The authors propose that IgA nephropathy is not mediated immunologically [59].

Observation of the sera of children with IgA nephropathy has revealed that the circulation of altered IgA induces the formation of macromolecular IgA aggregated by carbohydrate interactions and the formation of antigen–antibody complexes [60]. Such immune complexes could result from an immune reaction directed towards the aberrant carbohydrates. A deficiency in the terminal galactose has also been identified, and is believed to affect the clearance of the IgA from the circulation, through the formation of larger aggregates, which are not able to penetrate the fenestrae within the sinusoidal endothelial cells in the space of Disse [61]. Thus, the complexes can reach the glomerular circulation, where the fenestrae are five- to tenfold larger, and form deposits in the mesangium [61].

2.4. Alteration of clearance

A reduced clearance of the immunoglobulins or immunoglobulin fragments is sometimes involved in renal deposition diseases. Clinical studies with AL amyloidosis have shown that a 50% reduction in free light chain concentration (following chemotherapy) is associated with a substantial survival benefit [62], illustrating how a defective clearance could be a negative factor in the outcome of the disease. In the case of IgA nephropathy, the role of the liver in the clearance of IgA was first demonstrated in an animal model. After an IgA nephropathy was induced in mice by chronic immunization with dextran, a defective handling of IgA immune aggregates was demonstrated. Early impairment in the liver IgA clearance capacity is related to an increase in the deposition of immune complexes in the mesangium by raising the IgA aggregates concentration in the blood [63,64]. The impaired clearance and the involvement of the liver in IgA nephropathy were also demonstrated in humans [65]. Through the injection of ^{131}iodine-labeled aggregated IgA in patients as well as in healthy volunteers, the clearance of these aggregates was

shown to be significantly impaired in IgA nephropathy patients, compared to the healthy subjects [65]. Later experiments showed that these aggregates were removed from the circulation almost exclusively by the liver [66]. In another study, a mixture of heat-aggregated IgA and IgG was injected to human volunteers as well as IgA nephropathy patients. The liver was found to be predominantly involved in clearance, which was found to be defective in half of the patients with IgA nephropathy. Some splenic uptake was also observed [67]. One drawback of these studies is perhaps the use of immunoglobulin aggregates formed by heating. Upon heating, proteins undergo major denaturation and form aggregates that may not be equivalent to the mesangial deposits that are found in IgA nephropathy. Despite these limitations, the role of the liver seems likely in ensuring the elimination of immunoglobulin aggregates or immune complexes.

3. Fibril formation

Amyloid deposits have a fibrillar structure that can be observed by microscopy techniques such as electron microscopy and atomic force microscopy. Atomic force microscopy is able to image the growth of amyloid fibrils on surfaces, or immersed in aqueous media in near physiological conditions. According to Zhu et al., studying aggregation on surfaces is more relevant in the case of AL amyloidosis since the deposits in vivo are associated with surfaces [45]. Several groups have studied light chain fibrillation in vitro to elucidate the mechanisms of antibody fibril formation. However, the experimental conditions influence the formation of fibrils:

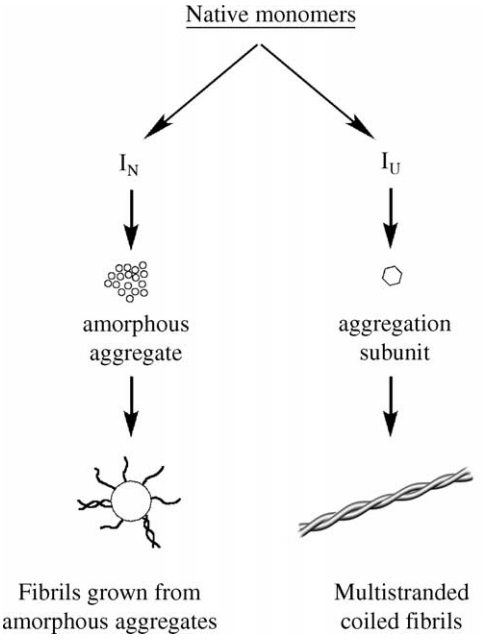

Fig. 2. Possible mechanism describing the fibrillation of the amyloidogenic light chain SMA, via partially folded intermediates. I_N is a native-like intermediate leading to amorphous aggregate formation. On a mica surface, fibrils grow out from these amorphous aggregates. I_U is an unfolded intermediate, which forms amyloid fibrils (adapted from Ref. [45]).

benign light chain LEN, which did not form amyloid fibrils in vivo, can be triggered to form fibrils in vitro under mildly destabilizing conditions [68,69]. The light chain Jto, which forms amorphous aggregates in vivo, also formed fibrils in vitro [70]. The molar ratio of Congo red, a dye used to stain amyloid deposits, can influence the type of aggregates (amorphous or fibrillar) formed by light chains [71]. Thus, extrapolation of in vitro results to in vivo situations should be done with care, especially when considering the potential activity of a drug against one of the immunoglobulin deposition diseases.

However, in vitro tests can provide a good insight into the nature of, and mechanism by which, the immunoglobulin species is involved in fibrillation. Most of the available data suggest that fibrils grow from proteins that adopt a non-native, aggregation-prone configuration.

An amyloidogenic light chain, SMA, was investigated to detect partially folded intermediates. In solution, two intermediates were observed, named I_N and I_U. I_N was shown to be relatively native-like and led predominantly to amorphous aggregates, whereas I_U, a compact but relatively unfolded intermediate, formed amyloid fibrils [72]. The same model, illustrated in Fig. 2, was applied to describe the two types of fibrils formed by the same immunoglobulin (SMA) on mica surfaces: each type of fibril came from a distinct intermediate, I_N or I_U [45].

An increased rate of fibril formation at low concentrations was observed in experiments on SMA as well as with the benign light chain, LEN. The entity responsible for fibrillation was proposed to be a monomer. In the organism, immunoglobulin light chains are present as dimers. In vitro near physiological conditions, light chains exist in a monomer–dimer equilibrium, meaning that at low concentrations the monomeric state will dominate [68,72].

The mechanism of fibril elongation is more controversial. Fink's group has proposed that oligomers serve as building blocks for the assembly of protofibrils [45,69]. This was contradicted by Takahashi et al. [73] where the polymerization of light chains isolated from five patients was better explained by a nucleation-dependent first-order kinetic model. The nucleation model implies that fibril elongation occurs through the addition of monomeric proteins onto the end of the growing fibril [73]. Differences in the experimental conditions may account for these contradictory results: SMA and LEN were recombinant light chains the fibrillation of which was triggered by agitation, whereas Takahashi et al. extracted the light chains from patients' organs and examined them without agitation [73].

One mechanism describing the fibrillation phenomenon is double nucleation (Fig. 3). Double nucleation and the subsequent assembly in higher-order structures such as multistranded cables are not exclusively associated with disease-related proteins. It has also been observed in human calcitonin, a therapeutic protein which tends to precipitate in solution, thus complicating formulation [74]. The human calcitonin fibrillation was explained by a double nucleation model with the protein monomers associated through an intermolecular

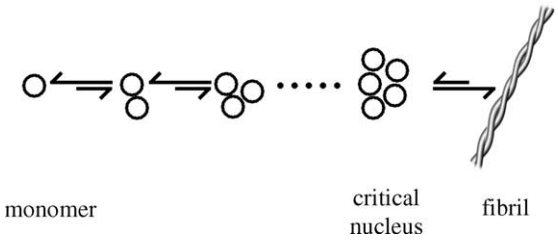

Fig. 3. Schematic representation of the double nucleation mechanism initial stages. Monomers randomly assemble into oligomers, until the oligomers reach a certain size and become critical nuclei. The equilibrium is then shifted towards protofibril formation.

β-sheet component [74]. The 4-nm-wide calcitonin protofibrils coil around each other to form helical fibrils and later cables or ribbons [75]. Similar observations have been made by atomic force microscopy on the amyloidogenic light chain SMA; 2.4 nm-wide protofibrils were shown to associate in a similar helical pattern to form thicker fibrils [76]. Understanding the fibrillation mechanism of a given protein can contribute to the improvement of formulation, especially when aiming at a long-term stable aqueous solution.

4. Biological effects triggered by aggregation

AL amyloidosis, LHCDD and IgA nephropathy are involved in renal failure. These three diseases affect the glomeruli. The exact mechanisms of toxicity, as well as the toxic species are not yet known with certainty. The toxic effects can occur on three levels: alteration of the mesangial matrix, direct action on the mesangial cell and stimulation of leukocytes leading to the generation of superoxide (O_2^-).

The presence of basement membrane components in amyloid deposits suggests that alterations of the basement membrane occur in AL amyloidosis. Immunolabelling studies performed on kidney biopsies from a patient with AL amyloidosis have revealed the co-localization of the following basement membrane proteins within amyloid deposits: serum amyloid P component, laminin and heparan sulfate proteoglycan core protein [77]. The absence of collagen type IV in amyloid was explained by a later in vitro experiment [78]. When mesangial cells were incubated with LHCDD light chains, an activation of TGF-β caused a decrease in collagenase IV activity, as well as an increased production of extracellular matrix proteins. In contrast, when mesangial cells were incubated with AL amyloidosis light chains, a decrease in TGF-β as well as a stimulation of collagenase IV occurred with a concomitant decrease in extracellular matrix proteins. Thus, in AL amyloidosis the mesangial matrix is replaced by amyloid fibrils, whereas in LHCDD the extracellular matrix is proliferating [78].

Peruzzi et al. have incubated mesangial cells with polymeric IgA, heat-aggregated IgA, IgA glycoforms and serum fractions from IgA nephropathy patients. The expression of integrins on the mesangial cell surface was then monitored by flow cytometry. Integrins mediate the cross-talk between mesangial cells and the surrounding matrix, and the expression of the $\alpha_v\beta_3$ integrin was shown to be up-regulated by macromolecular IgA and aberrantly glycosylated IgA [79]. The authors consequently proposed that integrin expression was modulating cell proliferation, apoptosis and phagocytosis of IgA deposits, factors playing an important role in the pathogenesis of IgA nephropathy. Mesangial cells would not seem to be passive in the pathogenesis of immunoglobulin aggregation diseases, but rather an active participant. This hypothesis was formulated a few years ago, for IgA nephropathy [80] as well as for AL amyloidosis [81].

The interaction between polymeric immunoglobulins and mesangial cells has been deduced from the observation of Fcα and Fcγ receptors at the cell surface [82]. In vitro experiments on rat mesangial cells illustrated that Fcα and Fcγ receptor occupancy triggered the synthesis and release of inflammatory cytokines (TNF-α and IL-6), cell proliferation, as well as the phagocytosis of the immunoglobulins. Heat-aggregated IgA and IgG induced a stronger response than the monomeric forms [82]. Similar experiments on human mesangial cells demonstrated that heat-aggregated IgA caused an overexpression of monocyte chemoattractant protein-1, IL-8 and IFN-inducible protein-10; an effect mediated by the Fc receptors for IgA (FcαR) [83,84]. However, even though both monomeric and aggregated IgA bind to human mesangial cells, the activation of the cell requires an aggregated form of IgA [85]. The activation of the cell was independent of FcαR1, which was explained by the existence of another IgA receptor on mesangial cells [85]. The presence of specific IgA receptor(s) on mesangial cells is supported by the induction of intracellular calcium mobilisation, DNA synthesis and cell proliferation, up-regulation of TGF-β mRNA and secretion of fibronectin observed upon aggregated IgA binding to cultured mesangial cells [86]. Mesangial cells have been shown to be directly activated by polymeric IgA via FcαR associated with the γ chain, which is a signalling subunit of Fc receptors [87,88].

The activation of mesangial cell by IgA aggregates, through FcαR, induces the synthesis and the release of various inflammatory cytokines, consistent with the observed increased renal infiltration of polymorphonuclear leukocytes in patients with IgA nephropathy. The leukocytes have a great potential for the production of reactive oxygen species, as was demonstrated by measuring H_2O_2 production after stimulation with heat-aggregated IgG [89]. The H_2O_2 production was significantly higher in the leukocytes from IgA nephropathy patients than from control leukocytes [89].

Furthermore, heat-aggregated IgA or IgG induced an activation of human neutrophils in vitro, leading to a greater superoxide production. Such activation might be mediated through FcαR; so that at least during the active phase of the disease, neutrophils are activated and play a role in the glomerular inflammatory process [90–92]. However, in vitro studies have shown that immobilized IgA induces neutrophil apoptosis, indicating that the deposits may exert a regulatory activity in the glomerular inflammation caused by IgA nephropathy [93].

5. Conclusions

Immunoglobulin aggregation, either in the form of amyloid fibrils or in the form of amorphous deposits, is strongly associated to fatal diseases: AL amyloidosis, LHCDD, cast nephropathy and IgA nephropathy. The mechanisms of immunoglobulin fibril formation have been the subject of much research. A consensus exists on the first aggregation steps, involving slightly destabilized intermediates. Several causes and consequences of impaired protein stability are summarized in Fig. 4. Amino acid substitutions can alter protein conformation and subsequently the protein aggregation state. At present, there is no general correlation between primary structure and aggregation. The in vitro aggregation of wild-type proteins emphasizes another aspect of protein stability: the influence of the local environment. The surface, the pH, the presence and concentration of ions, buffers or drugs can influence immunoglobulin aggregation, in vivo as well as in vitro. Aberrant glycosylation triggers self-aggregation in the case of IgA nephropathy. Immune complexes are also formed, and reduce the hepatic clearance of the immunoglobulins, raising their serum levels and increasing their potential deposition in the kidney. Immunoglobulin deposition has direct effects on the nephron, such as activation of mesangial cells, changes in the extracellular matrix and leukocyte infiltration.

The research on immunoglobulin deposition diseases provides insight into some of the causes of immunoglobulin aggregation. This research may have implications in the field of therapeutic protein production and formulation. In therapeutic proteins, the parameters influencing aggregation in vitro can be controlled. In many cases, modifications can be made in the protein sequence with beneficial effects on aggregation and minor changes in activity. Appropriate glycosylation may also improve the aggregation properties of therapeutic proteins. Finally, the formulation can control the local environment of the protein, before in vivo application. For new protein drugs, an open question will be the potential aggregation after in vivo injection, since the body can present a destabilizing environment to the protein, leading to aggregation. Further research could be focused on the analysis of therapeutic protein aggregates formed in vivo.

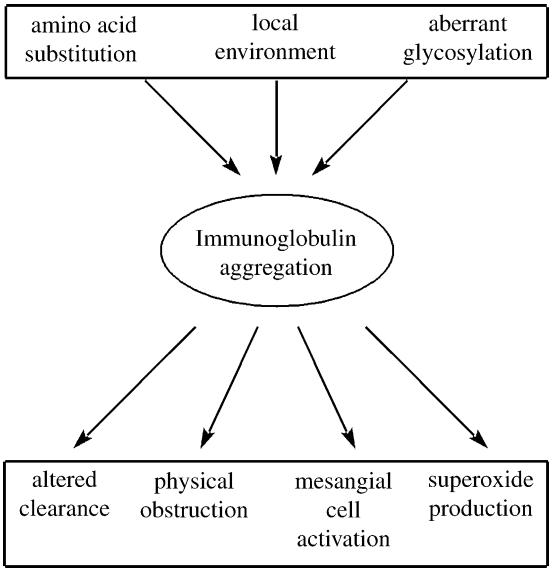

Fig. 4. Some causes (upper box) and potential consequences (lower box) of nephropathy-related immunoglobulin aggregation.

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