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GENETIC ANALYSIS AND THERAPY
OF PATIENTS AFFECTED BY FOCAL GLOMERULOSCLEROSIS

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Introduction

Focal glomerulosclerosis (GSF) is a renal disease characterized by segmentary sclerous lesions, which affect at first only some of the glomeruli, to then gradually spread to the whole renal parenchyma. The prognosis of GSF associated to nephrotic syndrome is unfavourable, with about 50% of the patients progressing towards dialysis in 5 years from the diagnosis. The etiopathogenesis of the disease is still largely unknown and the available treatments are often ineffective. Generally speaking though, it is possible to distinguish forms in which there is a genetic component, which leads to alterations in the structure and function of the basal membrane and of the podocyte, and so called “immunological” forms.

In the forms with a genetic component, defects have been described concerning the NPHS1 genes (nephrin), ACTN4, NPHS2 (podocin), CD2AP, WT1 and TRPC6, which encode important proteins for the functionality of the of the glomerular filtration barrier. Broader case histories report that about 45-55% of the recessive familial forms and 8-20% of patients with sporadic forms have mutations in the gene that encodes Podocin (NPHS2). Besides, a small percentage of patients with sporadic form are carriers of a mutation of the gene that encodes Nephrin (NPHS1). Mutations of other genes have been reported in literature as isolated cases.

The steroidal therapy, which is the first choice to treat patients with GSF, turns out to be ineffective in the majority of the cases, because these patients have a primitive alteration of the podocyte, and 50% of them inevitably progresses towards an end stage renal disease. Currently, renal transplant is the only possible alternative to dialysis for these patients. In these forms, the recurrence of the disease on the transplanted kidney is very rare (0.02%), if compared to the recurrence observed in all cases of GSF (30%).

On rats from the Imai strain, genetically susceptible to develop focal glomerulosclerosis, it has been found possible to reduce proteinuria and hyperlipidemy with drugs that act by blocking the renine-angiotensine system. Besides, a retrospective study on patients with diagnosis of GSF demonstrated the effectiveness of the Ace inhibitors in reducing considerably proteinuria. Therefore, a treatment with these drugs could be effective in reducing the renal damage n patients with a genetic form of GSF.

In the recent years, some evidences have come out to support the role of the immune system in forms in which a primitive alteration of the podocyte is not recognizable. It is in fact known that GSF is associated to the presence of atopy, lymphoproliferative diseases, and alteration of the TH2 immunity. Recently, a possible new pathogenetic mechanism of the GSF has been described. In some subjects affected by the disease, an increased expression of the CD80 at the level of the podocyte has been described. This molecule functions as a co-stimulating signal to activate the T lymphocytes and the immune response at renal level. This discovery gives an important link between the disease and the immune system, responsible in its turn of the damage at renal level.

Besides, some authors have supposed the existence of a circulating factor, which would increase the permeability of the glomerular filtration barrier, called permeabilizing factor (FP), produced by the T lymphocytes themselves and that could have a direct toxic effect on the podocytes. The existence of this factor is supported by the frequent recurrence of the disease, which happens a few days after the transplant in forms in which there haven't been identified mutations of known genes, and by observing that plasmapheresis can reduce proteinuria in many cases of recurrence of the disease. Nevertheless, this factor hasn't been identified yet. Besides, some data against the existence of the FP have recently appeared in literature, demonstrating the activity of the FP doesn't relate to variations of proteinuria in patients with GSF and the activity of the FP doesn't vary after supplying cyclosporine.

At the moment, it is supposed that the absence of a factor, important for the structural and functional integrity of the podocyte, is actually responsible for the increased permeability of the glomerular filtration barrier.

Steroids still represent the first therapy today for patients with non-genetic forms of GSF, with a response that considerably varies according to the duration of the treatment and to the dose supplied. About a third of the patients, though, is resistant to steroids or presents the recurrence of the disease as the therapy is stopped. A study on 49 patients with steroids-resistant GSF, randomized at the treatment with prednisone associated to placebo or cyclosporine, demonstrated that cyclosporine is able to induce remission of the nephropathy in 70% of these patients. Two years from the interruption of the therapy with cyclosporine, though, 60% of the patients presents the resumption of the disease. The use of cytotoxic agents as cyclophosphamide, clorambucil, or azathioprine has given even less encouraging results. Recently, a case of study of a child who has developed the recurrence of GSF after renal transplant has been published: in this case, a complete remission of proteinuria has been determined by the use of Rituximab (antibody directed against the B-lymphocytes antigen CD20), supplied to cure a secondary lymphoma from an infection due to the Epstein Barr virus. This case suggested a possible role in the pathogenesis of the disease for the B-lymphocytes, target of the therapy with Rituximab. At the moment, though, a strong rationale to support the use of this therapy to treat GSF doesn't exist.

During the last few years, it has been made available an antibody anti CD52, the Campath-1H, which is able to eliminate T-lymphocytes for more than 6 months. Given the alteration of the Th2 activity noticed in patients with GSF, it could be reasonable the treatment with this antibody. Nevertheless, the literature reported the case of a boy subjected to renal transplant where the therapy with Campath-1H didn't prevent from the recurrence of the disease. Besides, the lymphocyte deletion induced by this antibody could exposed the patients to a risk of opportunistic infections disproportionate to the disease that is meant to be cured.

Another approach potentially applicable to the cure of "immunological" forms of GSF could be to block the CD80 co-stimulating signals irregularly expressed on the podocyte, in order to prevent the activation of the T-lymphocytes. For this purpose, it could be possible to use compounds like Belatacept, already utilized to prevent acute rejection in renal transplant and to cure rheumatoid arthritis, but these drugs aren't available on the market yet and their safety profile hasn't been completely defined yet, because of the limited number of patients so far treated with it.

Aims of the study

Up till now, the absence of a clear knowledge of the pathogenetic mechanisms of the disease hasn't enable to find a targeted and effective therapeutic strategy to treat GSF. This research has been designed to improve the diagnostic criteria of the different forms of GSF, broadening our knowledge on the genetic causes of the disease. That will allow us to apply treatments that are specific and effective for the different forms of GSF, in order to slow down the progression of the disease.

Experimental plan

All the patients enrolled in the research will be at first treated with steroidal therapy (at the moment our sample includes 20 patients with sporadic form of GSF and 5 with the familial one). Among these, the ones who don't respond to the therapy with steroids will be typified for the defects in the known genes (NPHS1, ACTN4, NPHS2, CD2AP, WTI and TRPC6), with the methods of the DHPLC and the direct sequencing. Patients with mutations affecting these genes will enter in the Remission Clinic protocol, a strategy that includes dietetic rules, life style and therapy with ACE inhibitors, sartans and statins, aimed to reduce and slow down the progression of the nephropathy. This therapeutic strategy, already in progress in our Centre for 7 years till now, turned out to be effective in reducing the progress of the renal damage in all forms of nephropathies.

We expect though that more than 50% of our patients will not show any mutations in these genes. These patients will be treated with an innovative therapy that acts on the immunological component and implies the supply of thymoglobulin of rabbits (RATG), polyclonal antibodies human anti timocytes directed against lymphocytes T, B and the CD80. The logic in using RATG comes from the fact that they contain antibodies, which can potentially block all the immunological mechanisms responsible for GSF described so far. Besides, it has recently been demonstrated that the use of RATG remarkably reduces the risk of recurrence of GSF after transplant compared to Campath-1H and other monoclonal antibodies. A recurrent record of proteinuria and of the renal functionality parameters will assess the response to the treatment. Before and in different times after the treatment with RATG, we will also assess the frequency and phenotype of the leucocytic underpopulation in the blood (B and T lymphocytes, monocytes, natural killer cells and granulocytes). In particular, through flow cytofluorometry and quantitative PCR, we will study the expression of specific markers of activation (inflammatory cytokines, co-stimulation signals) and regulation (FOXP3, CTLA4, IDO, CD25).

Being impossible to exclude a genetic component from these “immunological” forms of the disease, in this group of patients we will look for possible alterations in new genes, by a study of conceivable genes, selected using the following criteria: genes that encode proteins involved in the regulation of the immune system; genes that could have a role preserving the morphology and physiology of the podocyte; genes that if missing or mutated cause the disease in animal models already described in literature. The methods of research used will be the analysis of linkage and the direct sequencing.

According to these criteria we will analyse:

Cytokines

Cytokines are polypeptides released under conditions of activation by different types of leucocytic cells, in particular T-lymphocytes; more than 20 have been described and some of them have a role of stimulation of the immune response (interferon γ , interleukin 2, tumor necrosis factor α , interleukin 12). An excessive production of these cytokines has been associated to the transplant rejection and to autoimmune diseases. Other molecules of this category though (interleukin 10 and interleukin 4) have a role in regulating the immune response and in “switching off” the activity of the lymphocytes.

Co-stimulation signals

They are molecules expressed on the surface of the T and B-lymphocytes, of the monocytes and of the dendritic cells. Some of them, like the CD80 and the CD86, are expressed under pathologic conditions in the podocytes and in other cells of the kidney, and can have a role in stimulating the inflammatory response and in inducing the renal damage.

Immune regulation markers

Together with the effector T-lymphocytes, responsible for the immune response, underpopulations of T-lymphocytes, specialised in regulating and stopping this response, have been described. These cells are characterized by the expression of intracellular molecules (FOXP3, IDO) and superficial molecules (CTLA4, CD25), which are essential for the regulating activity. For instance, FOXP3 knock-out (KO) mice develop a lethal poly autoimmune disease and the same happens in patients with mutations of this gene.

Integrins

Integrins are a family of heterodimeric molecules that mediate the attachment of the podocytes to the glomerular basal membrane and to the extracellular matrix. Alterations in the expression of the ITGA3 gene, which encodes the subunit α_3 , has been found in patients with membranous

nephropathy, suggesting the importance of the integrins in preserving the structure and functionality of the kidney. Kreidberg and colleagues have demonstrated that gene $\alpha3$ KO mice show renal anomalies typical of GSF.

ZO-1:

ZO-1 is a protein of the membrane that in the podocyte plays an important role as it bounds the peripheral actin filaments to the P-cadherin/catenin complex. An anomalous distribution of ZO-1 has been noted together with proteinuria in rats with spontaneous glomerulopathy.

The cadherin/catenin complex

Cadherins are adhesion cells that promote the interaction among cells and that bound themselves to the cytoskeleton through catenins. Many studies have put variations in the expression of cadherins in relation to processes like cell migration, cell proliferation, apoptosis and cell differentiation. These processes are potentially involved in the pathogenesis of glomerulonephritis.

The ILK-parvin complex

The ILK-parvin complex is important for the integrity and function of the podocyte as it acts as a support between the actin cytoskeleton and the glomerular basal membrane. Its destruction induces in fact matrix deposition, a decreased cell proliferation and apoptosis. Gene ILK deletion in the podocyte provokes a serious alteration of the glomerular basal membrane, albuminuria and focal glomerulosclerosis in murine models.

Nck1 and 2

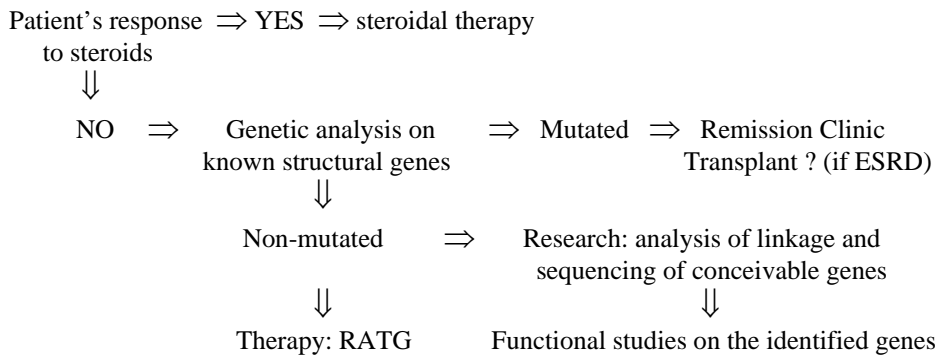
In all mammals, proteins Nck1 and 2 mediate the “communication” between receptors present on the cellular membrane and the actin cytoskeleton. A recent study has demonstrated that proteins Nck1 and 2 are both expressed in the podocytes, where they bound nephrin, and gene Nck1 KO mice with deletion of gene Nck2 in the podocytes develop albuminuria and focal glomerulosclerosis.

Once new genes related to the disease are found, we would be able to test the functional importance in animal models defective these genes (KO mice) and study the morphologic features of the podocytes in these animals.

The animal model will also serve studies of genic and cellular therapy:

1. We will try to typify the KO animal model
2. We will try to obtain hematopoietic stem cells from the bone marrow and we will verify their capability of colonizing the kidney and differentiating themselves in mature cells.
3. We will correct the defective gene in these cells through genic therapy approaches that imply using of many types of carriers containing the normal gene.
4. Permanently transfected cells will be selected, expanded and transfused in KO mice.
5. We will verify if these cells will be able to correct the defect at the heart of GSF.

The following chart sums up the experimental plan of the study:



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