Aicardi-Goutières syndrome. From study of the molecular pathogenesis to the development of therapeutic strategies in preclinical experimental models

Summary of research carried out on behalf of IAGSA in the period 2011-2015 at the Department of Health Sciences, University of Genoa, Italy

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Scientific background

Our research activity supported by IAGSA in the period 2006-2010 has made it possible to identify the characteristic gene expression profile that differentiates patients with Aicardi-Goutières syndrome (AGS) from control subjects (Izzotti et al., 2008). This, in turn, has made it possible to define the fundamental pathogenetic mechanisms of the disease, which consist primarily of interferon-alpha-induced activation of neurotoxic lymphocyte proteases (cathepsins) and arrest of brain angiogenesis (Izzotti et al., 2009). Indeed, inhibition of cathepsin D expression in vitro attenuates the neurotoxicity of AGS lymphocytes (Pulliero et al., 2012). These same studies also identified molecular mechanisms capable of arresting the clinical progression of the disease in patients aged over 3 years, namely activation of the lymphocyte inhibitor cystatin F and the interferon inhibitor DNAJ (Izzotti et al., 2009). Subsequent studies have identified the pathogenetic mechanism causing the overproduction of interferon-alpha in patients with AGS. This mechanism is a consequence of mutations in AGS-causing genes that silence the activity of endogenous ribonucleases. These enzymes are responsible for clearance of RNA sequences from the cytoplasm after their use. In AGS patients, this physiological process is impaired, resulting in accumulation of small fragments of RNA (microRNA); consequently, microRNA levels are increased in AGS patients compared with controls (Pulliero et al., 2011). This accumulation has the effect of blocking the production of the new microRNAs necessary for brain development, which is thus arrested. Finally, it has been shown in vitro that administration of neuroprotective microRNAs, whose production is blocked in AGS patients (miR-219), can attenuate the neurotoxicity of AGS lymphocytes (Pulliero et al., 2014). The build-up of microRNA in the course of AGS is detected by the intracellular sensors (TLRs) that in physiological conditions are responsible for the detection of abnormal nucleic acids, usually of exogenous viral origin. In AGS, however, it is the abnormal accumulation of endogenous microRNA that activates these sensors, triggering a response, similar to that which can be detected in infections by influenza-like RNA viruses, which leads to overproduction of interferon-alpha and starts the pathogenetic process of AGS (Pulliero et al., 2011).
Following the definition, by these studies, of the molecular pathogenesis of AGS, the next objective was to succeed in attenuating these mechanisms pharmacologically in \textit{in vitro} and \textit{in vivo} experimental models capable of reproducing them.

\textit{In vitro co-cultured cell models}

\textit{In vitro} models have been crucially important in performing initial assessments of drugs that may be of interest for modulating the pathological mechanisms of AGS. With the aim of reproducing, \textit{in vitro}, the molecular mechanisms underlying AGS we developed a system for co-culturing lymphocytes with astrocytes or vascular endothelial cells. In this situation, adding interferon-alpha causes lymphocyte activation. The resulting neutotoxic effects in the co-culture are detectable in the astrocytes, while the inhibitory effects on vascular growth are exerted on the endothelial cells. In this way, it proved possible to reproduce, \textit{in vitro}, the basic pathogenetic mechanisms of AGS. We performed a comparison between normal lymphocytes and lymphocytes taken from patients with AGS and demonstrated that the former, treated in the presence of high concentrations of interferon-alpha, can be used as surrogates for these experiments.

\textit{TES therapy}

We then evaluated the possibility of pharmacologically modulating the pathogenesis of AGS in the above-described \textit{in vitro} model. A specific software program (Literature Database Discovery) was used to identify the molecules theoretically capable of modulating the mechanisms concerned. These molecules were found to be: rapamycin, tacrolimus, sifalimumab (medi-545), erythropoietin, cystatin C, and cyclophosphamide. Cyclophosphamide was not evaluated due to its high toxicity. Sifalimumab is a monoclonal antibody against interferon-alpha, erythropoietin promotes vascularisation, cystatin is an inhibitor of lymphocyte activation, and rapamycin and tacrolimus are anti-inflammatory agents directed against mTOR-dependent mechanisms. Tacrolimus is a more modern analogue of rapamycin: it is effective, less toxic and has also shown neuroprotective effects. All these drugs were chosen because they are already used in clinical practice and because of their proven ability to cross the blood-brain barrier. The results obtained indicate that combining tacrolimus, erythropoietin and sifalimumab (TES therapy) is capable of effectively inhibiting the cytotoxic effects of lymphocytes activated by interferon-alpha. Indeed, neurotoxicity, measured as the loss of vitality of neuronal cells, was found to amount to 42\% in the presence of lymphocytes activated by interferon-alpha, whereas it was only 2\% when TES was added. Similar results have been obtained in relation to the growth of vascular endothelial cells: in the presence of TES, these
formed clusters; conversely, in the absence of TES these clusters were destroyed by lymphocytes activated by interferon-alpha (Izzotti et al., submitted).

Experimental animal models

To test the efficacy of TES in vivo it was necessary to develop a mouse model of AGS pathogenesis. Indeed, the currently available models (AGS knockout mice) are not sufficiently manageable in the experimental setting and moreover do not develop encephalitis but myocarditis. We therefore developed an experimental model based on the administration of oligonucleotides rich in guanine (G) and cytosine (C). These nitrogenous bases are indeed responsible for most TLR activation by small nucleic acids. The CG probes were encapsulated in lipid nanoparticle vectors able to ensure their intracellular delivery. In a first experiment, the CG probes were injected subcutaneously and their ability to activate lymphocytes in the spleen was determined cytofluorometrically. These experiments showed that 3 days after their administration the CG probes activated cytotoxic lymphocytes in the spleen; indeed, the percentage of cytotoxic lymphocytes in the spleen rose from 11% in the control to 28% in the treated animal. Administration of TES was found to be capable of inhibiting this activation, which dropped to 9% in the animal also administered CG probes.

Since the target organ in AGS is the brain, we then developed a model of intracranial CG probe delivery. For this purpose, we comparatively assessed different methods, determining how effectively they released, intracranially, fluid dyed with the tracer methylene blue. The following methods were assessed: slow-release administration via clot formation, use of an osmotic pump, and use of a flow pump (convection-enhanced delivery, CED). The most effective was CED. This method is based on application, in the experimental animal model, of the intracranial catheter used for the intracranial release of chemotherapy drugs in patients with brain tumours. The model was miniaturised to adapt it to size of the murine skull. An intracranial screw was inserted in the animal to allow repeated access to the catheter. The protocol was approved by the Committee for Animal Welfare before the experiment was carried out. We then proceeded with CG probe administration and three days later assessed the effect on intracranial activation of lymphocytes through flow cytometry of brain tissue. The results obtained showed increases, versus the saline only-treated controls, in the following parameters: (a) presence of interferon receptors on microglia (2% vs 10%); (b) percentage of microglial cells (1% vs 37%); (c) percentage of cytotoxic lymphocytes with receptors for interferon-alpha (1% vs 23%); (d) percentage of total lymphocytes (1% vs 7%). We also tested microglial activation through analysis of the CD45 receptors and demonstrated that their value increased twofold after administration of CG probes. This parameter is particularly relevant to
AGS as the microglia is the site of production of interferon-alpha in the brain. This experimental model therefore proved able, in the short term, to reproduce, in vivo, the basic pathogenetic mechanisms of AGS. We then performed long-term experiments with a duration of three weeks, administering CG probes intracranially on a weekly basis. The treated animals survived with neurological symptoms (opisthotonus, extrapyramidal rigidity, impaired gait) attributable to the cerebrotoxic effects of the CG probes. These symptoms recall the clinical situation of patients affected by AGS. We then determined the capacity of subcutaneous TES therapy to inhibit the pathogenetic mechanisms of AGS reproduced in the animal model through intracranial release of CG probes by means of CED, evaluating the modulation of the microglial activation. The TES therapy considerably reduced the magnitude of microglial activation by CG probes, bringing its values more into line with those of the vehicle-treated controls (Izzotti et al., submitted data). These results show that, in the animal model, TES can effectively modulate the AGS pathogenetic mechanisms even intracranially.

**ATM inhibitors**

Another finding of our research on gene expression in lymphocytes from patients AGS was that the overproduction of interferon-alpha leads to suppression of the expression of the gene coding for the recruitment of mesenchymal stem cells (DSC54). This activity has a key role in the development and growth of tissues. Its inhibition leads to an arrest of these processes inducing cell cycle arrest in stem cells, which instead should contribute to tissue development. We therefore hypothesised that, in AGS, mesenchymal stem cells may be blocked at the cell cycle arrest phase (G0) by the pathogenetic mechanisms of the disease. Today, drugs are known that are capable of overcoming this block: these drugs are used as radiosensitisers in radiation therapy for cancer. Indeed, cancer stem cells block their own cycle to escape the cytotoxic effects of radiation therapy. In order to overcome this mechanism of resistance, inhibitors of ATM, a key protein in cell cycle arrest at G0, were developed. We felt that it would be interesting to establish whether the use of these drugs might eliminate inhibition of neuronal growth caused by interferon-alpha-induced activation of lymphocytes. We extensively evaluated the toxicity of the ATM inhibitor KU-60019 on glial cells (in vitro) and mice (in vivo). No cytotoxic effects were found in vitro and in the in vivo evaluation no lesions were found in any of the organs studied (brain, haematopoietic marrow, heart, kidneys, liver, lungs, spleen, testes). We also assessed and ascertained the possibility of administering this drug to animals intracranially, by means of CED.

We then determined the ability of KU60019 to overcome the neuronal cell cycle block in vitro in co-cultures of lymphocytes activated by interferon-alpha and astrocytes. The experiments
conducted showed that KU60019 can overcome the cytokinesis block, allowing normal proliferation of neuronal cells despite the presence of cytotoxic lymphocytes activated by interferon. This line of research should now be carried forward, in order to establish whether the effects observed *in vitro* are also reproducible in experimental animals.

**Conclusions**

In conclusion, the results of the studies conducted show that effective modulation of the pathogenetic mechanisms of AGS is possible not only *in vitro* but also *in vivo* in the animal model. This modulation exerts direct effects on the target tissue of AGS, the brain.

**References**


