Abstract

Background

Leishmaniases is a group of diseases with clinical presentations ranging from self-healing cutaneous (CL) to destructive mucocutaneous (MCL), and visceral (VL) forms. VL represents a major health problem worldwide and the antileishmanial drugs used for its treatment have toxic side effects and the parasites have developed resistance to them therefore, new compounds and drug targets are sought. In this thesis I have probed *Leishmania* and leishmaniasis in biochemical and clinical studies. For the biochemical part, in one study I analysed the effect of the niacin analogue, 6-AN on *Leishmania* parasite growth and metabolism using the metabolomics technology. The rational was that, the pentose phosphate pathway (PPP), the major provider of antioxidants, has been reported as a target of 6-AN and *Leishmania* PPP enzymes have significant differences compared to those of the mammals, thus PPP might represent a good target. The other biochemical study was conducted to detect and characterize the *Leishmania* transglutaminase (TGase) enzymes that catalyze the irreversible protein crosslinking by creating chemical- and proteolytic-resistant isopeptide bonds within or between proteins/peptides. Therefore, if these enzymes are fundamental for *Leishmania* parasites, they could represent another promising drug target. TGase activity has been detected in some *Leishmania* species however, the protein TGase has not been purified, cloned, and characterized. The second part of the thesis is more clinical, evaluating in detail by advanced molecular techniques and cytokine analysis the prevalence of asymptomatic/subclinical *Leishmania* infections in autoimmune rheumatic patients treated with immunosuppressive biological drugs (anti-TNF-α antibodies, modulators of T lymphocyte activity, or anti-IL-6 receptor antibodies) and living in *Leishmania* endemic foci in Italy, thus representing a major disease re-emergence.

Methods

In the 6-AN study, *L. mexicana* M379 and *L. infantum* PCM5 promastigotes were treated with 7.8 mM 6-AN and 2.17% DMSO for 24 hours. After vitality, infectivity of 6-AN-treated promastigotes to mouse macrophages, and 6-AN interactions with oxidizing compounds were also studied. Small metabolites were extracted and analysed by pHILIC-LC-MS in polarity switching mode and data were analysed with IDEOMv19 and MetaboAnalyst 3.0.

In the TGase study, the enzyme activity was detected *in vivo* in canine *L. infantum* promastigotes after their incubation with fluorescein (FITC)-cadaverine (FC). Parasite smears were prepared, fixed and washed for 15 minutes at -20°C in methanol. A Nikon Microphot FXA fluorescent microscope was used to detect the specific incorporation of FC into parasite proteins. In addition, parasite extracts were incubated for 1 hour at 30°C with 4 mM FC, with or without dimethyl casein and the fluorescence was visualized in the SDS-PAGE gel using Molecular Imager Pharos FX imaging system (Bio-Rad). Further, TGase activity was measured in microwell plates using Sigma TGase activity assay Kit in parasite lysates and after precipitation with ammonium sulfate (AS). Western blot of human *L. infantum* promastigote whole lysate and canine *L. infantum* total extract and 15 and 45% AS protein fractions with TGase activity as well as immunocytochemical analysis of canine *L. infantum* were performed to detect *Leishmania* protein transglutaminase using rabbit polyclonal antibodies (pAbs, orb2986) against human TGase 2 and HRP-conjugated Goat anti-Rabbit antibodies or FITC-labelled anti-rabbit antibodies respectively. The blots were visualized by ECL system and the immunocytochemistry was revealed using a Nikon Microphot FXA fluorescent microscope.

In the clinical study, *Leishmania* qualitative PCR and real-time PCR were performed on DNA extracted from PBMCs from 50 autoimmune rheumatic (rheumatoid arthritis (RA), Ankylosing spondylitis (AS) and Psoriatic arthritis (PsA)) patients treated with immunosuppressive biologic drugs for at least 5 years. Genomic DNA extracted from *L. infantum* cultured promastigotes and from PBMCs from 50 healthy subjects were used as reference positive control and negative control respectively. Plasma cytokine concentrations were also measured in plasma from *Leishmania* DNA-positive and -negative rheumatic patients as well as from the healthy control group.

Results

In both *L. mexicana* and *L. infantum*, 6-AN caused significant depletion of phosphoribosylpyrophosphate (PRPP) and nicotinate (Na) and as a result purine and pyrimidine nucleotides were reduced and their nucleobases accumulated. Glutathione, ribose-5-phosphate, 6-phosphogluconate levels and downstream PPP intermediates were similar to controls. For *L. infantum*, it was possible to analyse NAD^+ and NADPH, which were found decreased together with the PPP

intermediate D-sedoheptulose 7-phosphate. Moreover, 6-AN treatment caused a marked elongation in parasite body. 6-AN in combination with the oxidizing compounds has additive effects against *Leishmania* and did not affect the infectivity of the treated promastigotes to mouse macrophages.

TGase activity was detected and confirmed in human and canine *L. infantum* promastigotes. Incubation of cultured promastigotes with FC resulted in a green intracellular fluorescing with clear non-fluorescing background indicating the presence of TGase activity in these parasites. In addition, incubation of promastigote lysates with FC and dimethyl casein revealed fluorescent endogenous as well as dimethyl casein protein bands in the SDS-PAGE gel confirming the presence of an active TGase. The canine protein TGase precipitated at 15 and 45% AS saturations and furthermore, the enzyme activity measured in 45% AS fraction using microplate assay was found Ca^{2+}-dependent and inhibited by 10mM GTP. The immunocytochemical analysis showed specific green fluorescence of detected TGase and the blots revealed calculated protein bands; one at position 74.6 KDa for the canine strain and two; a major one at position 55.34 KDa and a minor one at position 65.87 KDa for the human strain.

Eighteen out the 50 (36%) autoimmune rheumatic patients were positive for *Leishmania* DNA by conventional and/or quantitative PCR with a detection of high parasite burdens (1 to 136 parasite/ml in 4 patients, 1.000 to 40.000 in 11 patients and over 1.000.000 in 3 patients). Patients that were taking a steroid in association with the biological drug showed a higher positivity for circulating *L. infantum* kDNA than those given the biological drug only ($p<0.05$). No statistical difference observed in relation to the ownership of a dog and the type of biological drug administered. Pro-inflammatory IL-1, IL-6, IL-12(p70), IL-7, IL-15, IFN-γ and TNF-α; anti-inflammatory IL-4, IL-13; and regulatory IL-10 cytokines were markedly elevated in all autoimmune rheumatic patients with additional increases in inflammatory mediators in autoimmune rheumatic patients positive for *Leishmania* DNA.

Conclusions

In mammals 6-AN is converted to abnormal 6-ANAD/P by NAD^+ glycohydrolase, however, in *Leishmania* its toxicity is only seen in millimolar range, in which 6-AN is responsible for the depletion of cellular phosphoribosyl pyrophosphate (PRPP) content probably in the Preiss-Handler NAD^+ salvage pathway, resulting in

depletion of nucleotides required for nucleic acid biosynthesis. The marked elongation in the 6-AN-treated parasite bodies confirms nucleotide starvation. *Leishmania* NAD^+ glycohydrolase might decompose NAD^+ but might not catalyze exchange reactions, as found in other microrganisms, however, combined ^{13}C-glucose labeling and flux analysis might be useful to ascertain the fate and action mechanism of 6-AN in *Leishmania.* In addition, PRPP synthetase should also be a good target for new potential drugs against leishmaniasis pointing to the growth-inhibitory effect of PRPP depletion.

The presence of an active TGase in *Leishmania* was confirmed by detecting *in vivo* and *in vitro* a Ca^{2+}-dependent transamidation in canine *L. infantum* promastigotes. The inhibition of this TGase by GTP suggests that regulation of the activity of this enzyme might be Ca^{2+}/GTP regulated. Furthermore, the precipitation of the protein TGase at the low 15% AS saturation, suggests that this enzyme might be membrane-associated. In addition, the specific Western blots and immunocytochemical detections of protein indicate that the TGase pAbs (orb2986) could permit affinity chromatography purification of this *Leishmania* enzyme.

The high *Leishmania* parasitaemia detected in PBMC fractions from autoimmune rheumatic patients suggests that treatment with biologic drugs can lead to cryptic VL or *Leishmania* infection in a latent phase which may progress to full VL course in the setting of immunosuppression. In addition, taking a steroid drug in addition to a biological therapy is strongly associated with an increased risk of being positive for *L. infantum* kDNA. Molecular screening using the easy-to-obtain and-prepare PBMC fractions and cytokine analysis should be taken into account before treating autoimmune rheumatic patients with biologic drugs. Moreover, extension of this research even in rural and suburban areas may as well add new knowledge to map the spread of *L. infantum* infection.