Infection, Genetics and Evolution 49 (2017) 212–220

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Analysis of expression of *FLI1* and *MMP1* in American cutaneous leishmaniasis caused by *Leishmania braziliensis* infection

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ARTICLE INFO

Article history:
Received 29 October 2016
Received in revised form 10 January 2017
Accepted 18 January 2017
Available online 21 January 2017

Keywords:
Leishmaniasis
FLI1
MMP1
IL-6
Cutaneous lesions
Macrophages

ABSTRACT

FLI1 (Friend leukemia virus integration 1) and *IL6* (interleukin 6; IL-6) are associated with *Leishmania braziliensis* susceptibility. Cutaneous lesions show exaggerated matrix metalloproteinase 1 (MMP1). In other skin diseases, *FLI1* promoter methylation reduces *FLI1* expression, and low FLI1 down-regulates MMP1. IL-6 increases FLI1 expression. We hypothesized that epigenetic regulation of FLI1 in cutaneous leishmaniasis, together with IL-6, might determine MMP1 expression. While generally low (<10%), percent FLI1 promoter methylation was lower ($P = 0.001$) in lesion biopsies than normal skin. Contrary to expectation, a strong positive correlation occurred between FLI1 methylation and gene expression in lesions ($r = 0.98$, $P = 0.0005$) and in IL-6-treated *L. braziliensis*-infected macrophages ($r = 0.99$, $P = 0.0004$). *In silico* analysis of the FLI1 promoter revealed co-occurring active H3K27ac and repressive DNA methylation marks to enhance gene expression. FLI1 expression was enhanced between 3 and 24 hour post infection in untreated ($P = 0.0002$) and IL-6-treated ($P = 0.028$) macrophages. MMP1 was enhanced in lesion biopsies ($P = 0.0002$), induced ($P = 0.007$) in infected macrophages, but strongly inhibited by IL-6. No correlations occurred between FLI1 and MMP1 expression in lesions or infected macrophages (with/without IL-6). We conclude that MMP1 is regulated by factors other than FLI1, and that the influence of IL-6 on MMP1 was independent of its effect on FLI1.

1. Introduction

Leishmaniasis is a disease with a wide spectrum of clinical presentations, ranging from self-limiting lesions to severe mucosal disease. The magnitude of disease severity results from environmental factors such as parasite polymorphism, phlebotomine sandfly components, as well as the host's immune and genetic background. In particular, a number of studies (reviewed (Blackwell, 2010; Burgner et al., 2006; Castellucci et al., 2014)) have reported the role of host genetic factors in regulating the clinical disease outcome of *Leishmania braziliensis* infection in humans. The importance of the wound healing processes in cutaneous forms of leishmaniasis has also been demonstrated from studies mapping murine susceptibility genes (Sakthianandeswaren et al., 2010; Sakthianandeswaren et al., 2005; Sakthianandeswaren et al., 2009). In particular, fine mapping in the region of Chromosome 9 in mice (Chromosome 11q24 in humans) identified *Fli1* (Friend leukemia virus integration 1; *FLI1* in humans) as a novel candidate influencing both resistance to *L. major* and an enhanced wound healing response (Sakthianandeswaren et al., 2010). Recently we demonstrated (Castellucci et al., 2011) that polymorphism at *FLI1* is associated with cutaneous leishmaniasis (CL) caused by *L. braziliensis* in humans. We have also shown that the C allele at the *IL6*-174 G/C promoter polymorphism, which determines low levels of IL-6 release from macrophages, is a risk factor for mucosal leishmaniasis that occurs in some individuals following CL disease (Castellucci et al., 2006). IL-6 has been shown by RT-PCR to be expressed in lesions from both cutaneous and mucosal forms of American cutaneous leishmaniasis caused by *L. braziliensis* (Caceres-Dittmar et al., 1993), as well as in experimental cutaneous lesions caused by *L. major* infection (Karam et al., 2011). IL-6 is known to increase expression of *FLI1* (Thaler et al., 2011).

Recent RNA expression profiling (Novais et al., 2015) has demonstrated that the gene encoding matrix metalloproteinase 1 (*MMP1*) was amongst the most highly up-regulated genes in CL lesions caused

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http://dx.doi.org/10.1016/j.meegid.2017.01.018
1567-1348/

by *L. braziliensis* infection in our study area. This was of interest since reduction of FLI1 expression in human fibroblasts has been shown to result in down-regulation of MMP1, the authors of this work concluding that FLI1 suppression is involved in activation of the profibrotic gene program in fibroblasts (Nakerakanti et al., 2006). Heavy methylation of the CpG islands in the *FLI1* promoter region occurs in fibroblasts and skin biopsies, but not normal fibroblasts or skin, from systemic sclerosis patients (Wang et al., 2006), rendering *FLI1* transcriptionally inactive. Of interest too are recent observations that infection of macrophages with live *Leishmania* parasites is associated with changes in DNA methylation at hundreds of CpG islands (Marr et al., 2014), suggesting that the parasite might influence such epigenetic regulation of gene expression. The aims of the present study were: (i) to examine methylation and expression of FLI1 in normal and lesion skin biopsies from patients with CL; (ii) to determine whether *L. braziliensis* parasites influence methylation and expression of *FLI1* in macrophages infected *ex vivo*; and (iii) to determine whether or not changes in expression of FLI1 correlate with expression of MMP1 in lesion biopsies and/or infected macrophages.

2. Subjects and methods

2.1. Ethical statement

The study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines. The study was approved by the institutional review board of the Federal University of Bahia (CEP-UFBA 22/2012) and the Brazilian National Ethical Committee (CONEP: 1258513.1.000.5537). All patients provided written informed consent for the collection of samples and subsequent analysis. Parents or guardians provided informed consent for patients < 18 years of age. Patients between 12 and 17 years of age provided written assent.

2.2. Study site, diagnosis and sample collection

The study was conducted in the rural area of Corte de Pedra, Bahia, Brazil, where *L. braziliensis* is endemic. The endemic area of Corte de Pedra is characterized by isolated sites of secondary forest with agricultural activities providing the main source of income for the majority of its inhabitants. The work on farms and homes built in clearings in the woods has increased the population's exposure to *L. braziliensis* infection over the last decades (Queiroz et al., 2012). Participants of the present study include CL patients recruited at the health post in Corte de Pedra, the reference center for disease treatment of twenty municipalities. As in our previous studies (Castellucci et al., 2012; Castellucci et al., 2011; Castellucci et al., 2010; Castellucci et al., 2006) CL is defined as the presence of a single chronic ulcerative lesion at a skin site without evidence of mucosal involvement, without evidence of dissemination to 10 or more sites, and confirmed by detection of parasites or two of the three following criteria: positive delayed-type hypersensitivity test, PCR positive to *L. braziliensis*, and a histopathology consistent with leishmaniasis. Of the 31 patients used in this study, 65% (N = 20) presented with a single lesion, 16% (N = 5) with 2 lesions, 16% (N = 5) with 3 lesions, and 3% (N = 1) with 4 lesions. Full clinical details of these 31 patients are included under Supplementary information, which also provides details of the numbers of patients used in each type of study presented here, including the patient ID (far left column) which allows the reader to determine which pairs of samples were used in each type of study. All cases in the current study also responded to the standard anti-leishmanial therapy subsequent to samples being taken.

2.3. Biopsy specimens and DNA/RNA extraction

Biopsies of CL lesions (taken from the active leading edge at the lesion border) and normal skin (taken from a site remote to the lesion, usually another limb) from the same patient were taken using a 4-mm punch and tissues preserved in RNAlater Solution (Ambion) until the extraction of RNA and DNA. After intensive mechanical maceration using a syringe plunger, RNA and DNA were extracted for the gene expression and epigenetic regulation studies, respectively. RNA was extracted by using the PureLink RNA Mini Kit (Ambion) and DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen). The protocols recommended by the kit manufacturers were followed precisely. Both, RNA and DNA concentration and integrity were determined by spectrophotometric optical density measurement (260 and 280 nm) and samples stored at −70 °C.

2.4. Macrophages cultures and DNA/RNA extraction

Monocyte-derived macrophages (hereinafter referred to as macrophages) were prepared following a method previously shown (Giudice et al., 2012) by our laboratory to yield >99% macrophages characterized by flow cytometry as CD14-positive, CD3-negative, CD19-negative. Briefly, peripheral blood mononuclear cells were separated from blood over Ficoll hypaque (GE Healthcare Bio Sciences AB), and monocytes separated by adherence to plastic. Cells were maintained in Teflon vials in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (RP-10). After six days culture at 37 °C and 5% CO_2, differentiated macrophages were harvested by centrifugation, resuspended and allowed to adhere to glass coverslips for 24 h at 37 °C and 5% CO_2, in the presence or absence of recombinant human IL-6 (20 ng/ml) (Gibco). Following this incubation period, non-adherent cells were removed by rinsing, and adherent macrophages were cultivated in RP-10, and incubated at 37 °C, 5% CO_2.

Macrophage cultures were infected with *L. braziliensis* stationary phase promastigotes at a 5:1 ratio. Uninfected macrophages were used as controls. Infected macrophages were incubated for 2 h, after which time remaining extracellular parasites were removed by washing in HBSS. At each time point (3 and 24 h after infection), cells were harvested for DNA and RNA extraction. Cells used for DNA extraction were harvested in 50% fetal bovine serum, 40% RPMI-1640 (Gibco) and 10% DMSO and maintained at −70 °C. The remaining cells to be used for matched RNA extraction were harvested in TRIzol Reagent (Invitrogen) and also stored at −70 °C until use. DNA and RNA were extracted using the same kits and protocols described above.

2.5. Gene expression of FLI1 and MMP1

Reverse transcription reactions were performed using the commercially available High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), starting from 10 μl of total RNA and using the MultiScribe Reverse Transcriptase enzyme, according to the manufacturer's instructions. cDNA synthesis was carried out using the GeneAmp PCR System 9700 as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, finally cooling at 4 °C; and adjusted to 5 ng/μl. Assays containing specific primers and probes for *FLI1* (Hs00956709_m1) and *MMP1* (Hs00899658_m1) genes were pre-designed by Applied Biosystems®. The mRNA levels within each sample were normalized to the level of the house-keeping gene Beta-actin (Hs01060665_g1). The quantitative RT-PCR (qRT-PCR) individual reactions were prepared from 2 μl of cDNA, 0.5 μl of each assay, 5 μl of TaqMan Universal Mastermix® and 2.5 μl of RNase-DNase free water in a final volume of 10 μl. All samples were run in duplicate and the output level reported as the average of the two samples. Amplification conditions included: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min on the 7500 Real Time PCR System (Applied Biosystems). The data were analyzed by comparing the threshold cycle (Ct), according to the equation $2^{-\Delta\Delta CT}$ where ΔCT is the Ct value of the target gene subtracted from the Ct of the endogenous house-keeping gene, and ΔΔCT is the ΔCT value of each individual less the median ΔCT of the control group.