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Entamoeba histolytica: Inflammatory process during amoebic liver abscess formation involves cyclooxygenase-2 expression in macrophages and trophozoites

A. Gutiérrez-Alarcón [a], M. Moguel-Torres [a], O. Mata-Leyva [a], G. Cuellar-Nevárez [a], T. Siqueiros-Cendón [a], G. Erosa [a], E. Ramos-Martínez [b], P. Talamás-Rohana [c], B. Sánchez-Ramírez [a,*]

[a] *Laboratorio de Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Apdo. Postal 1542-C, Chihuahua, Chih., Mexico*
[b] *Departamento de Anatomía Patológica del Hospital Central Universitario, Facultad de Medicina, Universidad Autónoma de Chihuahua. Chihuahua, Chih., Mexico*
[c] *Departamento de Patología Experimental, Centro de Investigación y Estudios Avanzados del IPN, Mexico, D.F. Mexico*

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Abstract

It has been demonstrated that expression of cyclooxygenase-2 (COX-2) isoform is induced by *Entamoeba histolytica* in macrophages and polymorphonuclear cells during amoebic liver abscess (ALA) formation in hamsters. Trophozoites present in the lesion were also positive for COX-2 signal. However, no cross reactivity of the anti-COX-2 antibody with protein extract of cultivated trophozoites was found. To clarify if trophozoites are involved in PGE_2 production during ALA development, COX-2 expression was detected by in situ hybridization and RT-PCR in liver tissue from intrahepatically infected hamsters. COX-2 mRNA was in polymorphonuclear cells since 4 h postinfection, and subsequently, local macrophages expressed COX-2 mRNA in a similar way. Additionally, a positive signal for COX-2 mRNA expression was detected in *E. histolytica* trophozoites, suggesting that, in vivo, parasite COX expression may be an important mechanism to promote inflammation.
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Index Descriptors and Abbreviations: Entamoeba histolytica; Amoebiasis; Inflammation; Cyclooxygenase; Prostaglandins; ALA, amoebic liver abscess; COX-2, cyclooxygenase-2; IHC, immunohistochemical analysis; ISH, in situ hybridization; MOs, macrophages; p.i., postinfection; PGs, prostaglandins; PGE_2, prostaglandin E_2; PMNs, polymorphonuclear leukocytes; RT-PCR, reverse transcription and cDNA amplification by polymerase chain reaction

1. Introduction

During amoebic liver abscess (ALA) formation, macrophage activity is probably suppressed by inflammation-derived mediators, such as prostaglandins (PGs) (Wang and Chadee, 1992). Conversion of arachidonic acid to PGE_2 is catalyzed by a cyclooxygenase (COX; also known as prostaglandin endoperoxide synthase, EC 1.14.99.1) enzyme. In a hamster model of ALA, early stages of liver lesions are characterized by an acute inflammatory response which progresses rapidly to become granulomatous within 4 days postinfection (p.i.). Polymorphonuclear leukocytes (PMNs) are the predominant cell type found early in the infection, afterwards tissue is infiltrated around the lesion site by macrophages (MOs) and lymphocytes (Tsutsumi et al., 1984). We have demonstrated that development of experimental ALA curses with an increase in both, systemic levels of PGE_2 and local COX activity in liver microsomes. In addition, treatment of infected hamsters with Indomethacin, a nonspecific COX inhibitor, reduced ALA formation by 30% (Sánchez-Ramírez et al., 1997).

At least two isoforms of this enzyme have been described; COX-1 a constitutive enzyme and COX-2 an

* Corresponding author. Fax: +52 614 414 44 92.
E-mail address: bsanche@uach.mx (B. Sánchez-Ramírez).

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inducible isoform associated with inflammation (Holtzman et al., 1992). Experimental evidence has shown that COX-2 protein is expressed in vitro by ALA derived macrophages (AMAs) (Sánchez-Ramírez et al., 1998). Additionally, immunohistochemical analysis (IHC) of infected livers, showed induction of COX-2 protein in AMAs (Sánchez-Ramírez et al., 2000, 2004), and in MOs present in lamina propria of infected human intestine (Stenson et al., 2001). Also, we have found the presence of COX-2 in ALA trophozoites but not in cultured trophozoites. The expression of COX-2 mRNA was detected in infected liver at 2, 4, and 7 days p.i., which suggest that PGE_2 biosynthesis during *Entamoeba histolytica* infection involved de novo synthesis of COX-2 protein (Sánchez-Ramírez et al., 2000, 2004). Recently, Dey et al. (2003) isolated and characterized a cyclooxygenase-like enzyme from *E. histolytica* (registered as actinin-like protein with GenBank Accession No. AAF20148.1), whose activity in trophozoites is increased, in vitro, by addition of exogenous arachidonic acid.

To analyze if COX-2 expression in trophozoites was due to interaction of the parasite with host's tissue, or to ingestion of cellular detritus by the parasite, we determined the presence of COX-2 protein by IHC, and the expression of COX-2 mRNA by in situ hybridization and RT-PCR in infected livers at 4, 8, and 12 h p.i., and 4 and 7 days p.i.

2. Materials and methods

2.1. Chemicals and reagents

Absolute methanol, bovine serum albumin, Denhardt's solution, dextran sulfate sodium salt, diethyl pyrocarbonate, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), formamide, hydrogen peroxide, magnesium chloride, paraformaldehyde, potassium chloride, sodium citrate, sucrose, Tris–HCl, and yeast tRNA from Sigma-Aldrich (Toluca, México). Oligonucleotides primers were synthesized at Bio-Synthesis (Lewisville, TX, USA). SuperScript II-reverse transcriptase (of Moloney murine leukemia virus), water (RNAse free), proteinase K, and *Taq* DNA polymerase recombinant from Invitrogen (Rockville, MD, USA). d-Nucleotide solution from New England Biolabs Inc. (Beverly, MA, USA). Centrisep column from Princeton Separations (Adelphia, NJ, USA). PCR purification system from Marligen Bioscience (Ijamsville, MD, USA). DIG DNA Labelling and Detection Kit, PCR DIG labeling Mix, and oligo-$(dT)_{15}$ were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Tween 20 from Bio-Rad (Hercules, CA, USA). For IHC analysis, anti-COX-2 antibody was from Cayman Chemical Co. (Ann Arbor, MI, USA). Histostain-plus kit (Zymed Lab. Inc. San Francisco, CA, USA). Xylene and ethanol from J.T. Baker (México State, México). Entellan resin mounting medium was purchased from Merck (México State, México).

2.2. Animals and infection procedure

Male inbred hamsters (*Mesocricetus auratus*) weighing approximately 100 g were infected intrahepatically with 1×10^6 trophozoites as described previously (Sánchez-Ramírez et al., 1997). At 4, 8, 12, and 24 h p.i., and 2, 4, and 7 days p.i., animals were anesthetized and killed by exsanguination, and livers and abscesses removed and fixed with 4% phosphate-buffered formalin and paraffin-embedded.

2.3. Immunohistochemical analysis (IHC)

To corroborate the presence of COX-2 protein in tissue sections, IHC was performed as described previously (Sánchez-Ramírez et al., 2004). Briefly, liver samples were rehydrated, and equilibrated in phosphate-buffered saline bath. IHC was performed with Histostain-plus kit following the manufacturer's instructions. The primary antibody, polyclonal rabbit anti-COX-2 antiserum (1:250 dilution), was detected using the secondary affinity-purified biotinylated goat anti-rabbit IgG antibody, avidin-peroxidase, and freshly prepared diaminobencidine substrate.

2.4. COX-2 cDNA probe synthesis

Total RNA was isolated from normal hamster kidney and cDNA was generated by conventional RT-PCR in tube, as previously described (Sánchez-Ramírez et al., 2004). The following primers for COX-2 were used: sense primer 5′-CTG TAT CCC GCC CTG CTG GTG-3′, antisense primer 5′-ACT TGC GTT GAT GGT GGC TGT CTT-3′ (Badawi and Archer, 1998). PCR primers were designed based on the homologous rat COX-2 gene (GenBank Accession No. S67722) corresponding to nucleotides 1588–1880 (Feng et al., 1993). The amplification product of 285 pb was purified using a PCR purification system (Marligen Bioscience) and quantified by densitometry with a laboratory imaging GDS-8000 Chemi System and a LabWorks 3.0 software (UVP, Upland CA, USA). For in situ hybridization, the probe was labeled with digoxigenin-deoxyuridine-triphosphate (DIG-11-dUTP) by the random primed DNA labeling technique, using the DIG DNA Labeling and Detection Kit. A 10 ng aliquot of the PCR product thus generated was used in a second 25 cycle PCR reaction with a set of COX-2 primers and product was purified with a Centrisep column, and sequenced in both strands in an Abi Prism Genetic Analyzer model 310 (Applied Biosystems). Sequence alignments were generated for the COX-2 fragment using default options in CLUSTALX, and compared with the nucleotide sequence of rat COX-2, and the actinin-like protein from *E. histolytica* (GenBank Accession No. AAF20148) (Dey et al., 2003). The resulting nucleotide sequence is shown in Fig. 1. Sequence search was done using the BLASTX 2.2.13 (Altschul et al., 1997) to identify putative COX related genes in the *E. histolytica* genome (TIGR data base).

2.5. Nonradioactive in situ hybridization (ISH)

COX-2 mRNA expression was detected in paraffin-embedded tissue samples from normal or infected hamster by

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