MOLECULAR GENETIC MAPPING OF COSTELLO SNYDROME AND HEME OXYGENASE-1 GENE EXPRESSION STUDIES IN PEDIATRIC PATIENTS

Summary of Ph.D. thesis

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Introduction

Costello syndrome

Costello syndrome is a rare congenital disorder characterized by postnatal growth deficiency, mental retardation, "coarse" face, loose skin of the neck, hands and feet, cardiomyopathy, and nasal papillomata. The first cases were described in 1971 and 1977 by Costello et. al. To date, the genetic basis of Costello syndrome is unknown. Autosomal dominant inheritance with de novo mutations has been suggested by Lurie [1994] to explain the usually sporadic occurance of the syndrome. A first clue to map the gene was provided by Costello patient carrying an apparently balanced chromosomal translocation t(1;22) (q25;q11) [Czeizel and Timar, 1995].

Oxidative stress in neonates

Birth itself causes mechanical and oxidative stress for newborns. All infants, regardless of gestational age, have shown evidence of oxidative stress during the first few days after birth, especially premature infants, who have much lower antioxidant capacity than term babies. The switch to aerial breathing, the increased partial O_2 concentration and the stress caused by birth induces many physiological processes, most notably the neonatal adaptation to the greatly changed conditions.

Part of the adaptation is the rapid degradation of fetal hemoglobin and the oxidation of its heme moiety by heme oxygenases (HO), which are contributing factors in the development of post parturition hyperbilirubinemia. The released free heme is an aggressive oxidative agent which can be directly cytotoxic and it can also become toxic by mediating oxidative stress and inflammation.

Oxidative stress in hemodialysed patients

The principal causes of morbidity and mortality in patients with end-stage renal disease (ESRD) requiring long-term hemodialysis (HD) are cardiovascular complications. Even as adolescents these patients have rapidly progressing arterial diseases, arteriosclerosis, and atherosclerosis. Previous experimental and clinical studies have highlighted the role of leukocytes in generating reactive oxygen metabolites as a primary mechanism of oxidative stress during each HD session. In uremic patients the changes in Hb metabolism are the major cause of HD-mediated endothelial injury.

Role of heme oxygenases

Heme oxygenases catalyze the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, which is subsequently converted to the antioxidant bilirubin by biliverdin reductase. Biliverdin and bilirubin are potent antioxidant themselves and play a protective role against further oxidative stress. The transiently enhanced heme oxygenase-1 (HO-1) mRNA accumulation is a sensitive marker of oxidative stress and the induced HO-1 plays a cytoprotective role in oxidative stress and heme-mediated injury.

HOs may have a pro-oxidant effect, as the iron released from heme (in the setting of low ferritin availability) may react with hydrogen peroxide via the Fenton reaction to form hydroxyl radicals. On the other hand producing biliverdin and its metabolite bilirubin, however, HOs may act with an antioxidant effect, because bilirubin is a potent antioxidant. The effect of HOs on oxidative cell injury may vary considerably with cell type, level of expression, and chemical properties of the oxidant. Furthermore, HOs convert a lipid-soluble oxidant (heme) into iron, which can be sequestered with ferritin, when available. This protects the cell from lipid peroxidation that would otherwise occur, particularly considering the affinity of heme for the hydrophobic cell membrane interior.

Aims

1. To date, the genetic basis of Costello syndrome is unknown therefore our aim was to refine the break point regions of a Costello syndrome patient with an apparently balanced chromosomal translocation t(1;22) (q25;q11) at the molecular level by FISH.
2. There was a need to establish a scaleable and sensitive method to measure the HO-1 expression because of the limited amount of samples available from premature neonates.
3. Although HO-1 induction may be a general and adaptive response to oxidant stress, the inducibility of this enzyme in the early postnatal period in human newborns had not been studied yet. We hypothesized that enzymatic immaturity of HO-1 or its regulation system could play a role in the early transitory adaptation disturbances of premature neonates. Therefore our aim was to investigate the HO-1 expression in mature and premature neonates during the fist week after birth to find out if the enzyme is inducible and whether there are any differences between the two groups.

4. The changes in Hb metabolism in uremic patients are the major cause of HD-mediated endothelial injury. Therefore our aim was to follow the effects of single HD sessions on the plasma hemoglobin and bilirubin as indicators of hemolysis, the ferroxidase activity, the erythrocyte-derived reduced and oxidized glutathione levels and HO-1 mRNA expression as oxidative stress markers, and the homocysteine levels as and independent risk factor.

Methods and patients

Methods used in the positional cloning experiments

The selected yeast strains containing the YAC inserts were cultured according to Burke et al. PAC, BAC, and cosmid cultures were grown in 2ml of LB medium in the presence of the appropriate antibiotic at 30°C until an OD600 of 0.6 0.8 was reached.

Isolation of the DNA insert from YAC, PAC, BAC and cosmid clones

The protocol to separate yeast DNA from the culture was based on that of Philippsen et al. The obtained yeast chromosomes were run on a 1.2% low-melting-point agarose gel by pulse field electrophoresis. Gels were stained for 15 minutes with ethidium bromide and subjected to UV light for visualization of the DNA. The extra bands containing the YAC insert were cut out by scalpel. The DNA was isolated from the low-melting-point agarose and purified. For PAC, BAC and cosmid clones DNA was prepared from 2 ml of culture using a standard alkali miniprep protocol.

Fluorescence in situ *hybridization (FISH) experiments*

The chromosomes and nuclei spread was prepared from metaphase stopped fibroblast culture of the patient as described in Kutsche et al. The probes were nick translated with biotin-dUTP. The YAC, PAC, BAC, cosmid probes were labeled by fluorescein isothiocyanate. Additionally a TEL 1q DNA probe labeled with Texas red was used to visualize chromosome 1. The chromosomes were counterstained with 4',6-Diamidino-2-phenylindole.

Methods used in the gene expression experiments

RNA extraction and competitive reverse transcriptase (cRT)-PCR experiments

The Human DNA sequence from clone CTA-286B10 on chromosome 22 was used to design the primers for the experiments. Primers were designed to span exon boundaries to avoid binding of primers at DNA level. The amplicon and primer binding sites were checked by RepeatMasker and BLAST-ed to database 'nr' to avoid designing primers into repeat regions or other conservative protein motifs.

The mRNA was extracted from 1 ml (100μl in case of neonates) of venous blood with a magnetic particle mRNA Isolation Kit. Competitive reverse transcriptase PCR was used to identify the expression of heme oxygenase-1 gene. The competitor RNA was created by in vitro mutagenesis from HMOX1 cDNA and transcription with T3 RNA polymerase according to Waha et al. The primers to generate the competitor were as follows:

HMOX1-T3 5′ AAT TAA CCC TCA CTA AAG GGA GAC GTT TCT GCT CAA CAT CCA GCT C 3′

HMOX1-mut 5′ CCT GGG AGC GGG TGT TGA GTG GGG GGC AGA ATC TTG CAC TTT G 3′

First-strand cDNA was generated by using the RevertAid First Strand cDNA Synthesis Kit with the specific primer of HMOX1-R. The PCR amplification was carried out with the following program: initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 20 s, annealing at 61°C for 30 s and extension at 72°C for 20 s, followed by a final extension at 72°C for 10 min. The primers were as follows:

HMOX1-F 5′ CGT TTC TGC TCA ACA TCC AGC TC 3′

HMOX1-R 5′ CCT GGG AGC GGG TGT TGA GTG 3′

The amplified cDNAs were examined on 6% polyacrylamide gels and stained with ethidium bromide. The target HMOX1 band was calculated by the ratio to the competitor by densitometry. HMOX1 mRNA concentrations were expressed with reference to the white blood cell count.

Biochemical methods

Blood Hb concentration was measured with an OSM 3 hemoximeter. For the assay of plasma Hb and its oxidized metabolites, metHb and hemichrome, heparinized plasma samples were measured spectrophotometrically at different wavelengths. Plasma concentrations of the unbound Bi were estimated by spectrophotmetric method of Jacobsen and Wennberg. The total ferroxidase activity and the ceruloplasmin ferroxidase activity in the plasma were measured with conalbumin. Homocysteine concentrations were measured by HPLC.

Reduced (GSH) and oxidized glutathione (GSSG) concentrations were measured separately in the whole blood by a combination of previously accepted standard methods, and were expressed with reference to Hb determined by the cyanmethemoglobin method.

Characterisation of the Costello syndrome patient

We analyzed the Costello syndrome patient (a Hungarian girl) with an apparently balanced translocation 46,XX t(1;22)(q25;q11) described by Czeizel and Timar. Cultured metaphase stopped fibroblast cells were used for the FISH experiments.

Characteristic of mature and premature neonates

We analyzed 21 mature (gestational time are 37-40 weeks, birth weight: median 3305g; quartiles 3060g, 3770g) and 20 premature neonates (gestational time are 26-36 weeks, birth weight: median 1860g; quartiles 1450g, 2230g) with transient neonatal adaptation difficulties.

Characteristic of HD patients

17 HD patients dialyzed in our dialysis unit were included in the study. The duration of HD treatment in these 17 patients was 38 months (median, quartiles: 16; 102). The patients were grouped according to the duration spent in the dialysis program: short-term HD patients (n=7, median 19 months, quartiles: 9, 29) and long-term HD patients (n=10, median 97 months, quartiles: 53, 150).

Results

FISH mapping of the apparently balanced translocation Costello syndrome patient

We re-analyzed the chromosomes of this translocation by high-resolution banding (450- to 550-band level) that confirmed the breakpoint on chromosome 1q25, and refined that on chromosome 22 to q12. In order to delineate and define the breakpoint regions at the molecular level, FISH analysis was done by YAC, PAC, BAC and cosmid clones from the corresponding chromosomal regions.

By FISH analysis of several YAC clones we mapped the breakpoint region between the STS markers of D1S2078 and SOAT1. We found that YAC 790F06 which contained these markers and spanned the breakpoint region. Subsequently, 43 PAC clones were isolated and 14 placed in order to form a partial contig using microsatellite and end-sequence STS markers derived from selected PAC clones.

The breakpoint on chromosome 22 was originally localized by conventional cytogenetic analysis and assigned to 22q11. Since reinvestigation by high-resolution banding suggested that the breakpoint was in q12, we performed FISH experiments by YAC clones from 22q11-q12. Two clones, 765E02 (500 kb), and 881H10 (650 kb) hybridized proximal to the breakpoint suggesting that the breakpoint region is more telomeric. Similarly, seven additional YAC clones, cytogenetically assigned to 22q11.2 to q13.1, hybridized proximal to the breakpoint. In contrast, two YACs, 803D03 and 924C02, mapped to the end of 22q13.1 and the beginning of q13.2, hybridized distal to the breakpoint. ased on this data, FISH analysis refined the cytogenetic breakpoint from 22q11 to 22q13.1. No YAC clone was found located in the putative breakpoint region. Instead, numerous PAC, BAC, and cosmid clones have been used for further FISH experiments. Of these, three PAC/BAC clones, CTA-150C02, RP3-494G10, and RP4- 742C19, hybridized proximal and two, CTA-234D04 and RP3-333H23, distal to the breakpoint, whereas cosmid LL22NC03-10C3 spanned the breakpoint.

Estabilished a sensitive cRT-PCR method

We created our control RNA fragment from reverse transcribed cDNA of HMOX1 mRNA by *in vitro* mutagenesis. The control RNA contained a 20 bp internal

deletion, and had the same amplification characteristic that HMOX1 mRNA both at the RT and PCR steps. We verified the specificity of our amplified target by restriction endonuclease digestion, we also verified that our primers did not bind at the genomic level and that the amplification was not disturbed by genomic DNA contamination.

HO-1 expression in mature and premature neonates

The relative HO-1 mRNA levels and inducibility of the mature and premature neonates proved similar. HO-1 expression was induced on days 2 and 3. Later, the HO-1 mRNA levels decreased and dropped below the day 1 value by the end of the first week. HO-1 expression was induced on days 2 and 3. Later, the HO-1 mRNA levels decreased and dropped below the day 1 value by the end of the first week. The serum bilirubin levels of the mature and premature neonates increased significantly after birth in both groups, and were the highest on day 5 after birth.

HO-1 expression in young hemodialysed uremic patients

Our study, which monitored the alteration in the HD-associated oxidative stress after one HD session, revealed significant differences in HO-1 inducibility, depending on the duration of HD treatment. In those patients who had been on HD for a shorter duration, the baseline HO-1 mRNA expression was higher than in those undergoing long-term HD. In the short-term patients, the plasma Hb level did not increase significantly during one HD session, because of the already elevated HO-1 level, and HO-1 was not further induced. In the long-term HD patients, the baseline HO-1 mRNA expression was low and the HO-1 expression was upregulated one- to five-fold during HD, due to the ensuing hemolysis.

To estimate the effects of HD on the different parameters, the ratios (after HD)/(before HD) of the measured metabolites and the HO-1 mRNA levels were correlated. Significant correlation were found between the change in HO-1 mRNA level and the changes in plasma Hb level ($r=0.72$, $P<0.001$) and plasma Bi level ($r=0.71$, $P<0.002$).

Conclusions and original findings

1 In conclusion, we mapped the translocation breakpoint on chromosome 1q25 to a 109 kb region. The breakpoint on chromosome 22 was refined to q13.1 and a cosmid with an insert of 38 kb was found that spanned the breakpoint.

2 We established a robust, sensitive molecular genetic approach to measure directly the HMOX1 mRNA expression. The method was capable to measure small amount of whole blood samples (100µl), and was insensitive of inhibitors of either the RT or the PCR amplification steps.

3

a In our experiments with HO-1 expression in healthy mature and premature we found that level of HO-1 expression and its induction profile are similar in both mature and premature neonates during the first week after birth.

b We also showed that HO-1 is functional in both mature and premature neonates because the induction of HO-1 was followed by the increase of the bilirubin levels. Thus in healthy neonates HO-1 does not play a role in the transitory adaptation disturbances however it has an important role in the physiological adaptation process. Our study revealed the importance that further analysis is needed to analyze the HO-1 expression in neonates with organic manifestation of oxidative injury.

4

a In HD patients we showed that there is a significant difference in the HO-1 expression pattern between the patients depending on the duration of HD treatment. Short-term HD patients have an elevated HO-1 expression which may be contributed to an ongoing inflammation process. Long-term HD patients have a low base line HO-1 expression which was up regulated 1-5 fold during one single HD session.

b We also showed that the induction of HO-1 significantly correlated ($P<0.001$) with the occurring hemolysis and the liberated heme. We concluded that the periodical oxidative injury (three times a week) due to every HD sessions and the time delay between the occurring hemolysis and up-regulation of HO-1 could also contribute to the accelerated atherosclerosis rate in long-term HD patients.

5 Articles related to the thesis

I. Maróti Z, Kutsche K, Sutajova M, Gal A, Nothwang HG, Czeizel AE, Timar L, Solyom E.: **Refinement and delineation of the breakpoint regions of a chromosome 1;22 translocation in a patient with Costello syndrome.**
Am J Med Genet. 2002 May 1;109(3):234-7.
PMID: 11977185

II. Zoltán Maróti*, Sándor Túri, Ilona Németh, Eszter Karg, Péter Ugocsai, Emőke Endreffy: **Heme oxygenase 1 (HMOX1) gene expression in hemodialysed uremic patients.**
Acta Biologica Szegediensis, 47(1-4):147-151, 2003

III. Maróti Z, Németh I, Túri S, Karg E, Ugocsai P, Endreffy E: **Heme oxygenase 1 expression in young uremic patients on hemodialysis.**
Pediatr Nephrol. 2004 Apr;19(4):426-31. Epub 2004 Feb 24.
PMID: 14986081

IV. Farkas Ildikó, Maróti Zoltán, Katona Márta dr., Orvos Hajnalka dr., Németh Ilona dr., Endreffy Emőke dr., Pál Attila dr., Túri Sándor dr.: **Hemoxigenáz-1 génexpresszió érett és koraszülött újszülöttekben**
Magyar Nőorvosok Lapja [in press]