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Assessment of the genotoxicity of atenolol in human peripheral blood lymphocytes: Correlation between chromosomal fragility and content of micronuclei

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ABSTRACT

The antihypertensive drug atenolol was found to induce chromosome loss, detected as micronuclei in the peripheral lymphocytes of treated patients. The fundamental question which chromosomes the micronuclei were derived from remains to be answered. Analysis of structural chromosomal aberrations (CAs) and expression of fragile sites (FS) were pursued in this study. They revealed a significantly higher incidence of chromosomal aberrations (chromatid and chromosome breaks) in patients compared with controls, where 10 FS emerged as specific. Also, the band 17q12–21, where known fragile sites have not been reported, was only expressed in atenolol-treated patients. Fluorescence *in situ* hybridization using chromosome-specific probes revealed the preferential involvement of chromosomes 7, 11, 17 and X in the micronuclei (MN) of patients. The results also suggest a correlation between chromosomal fragility and content of MN, and support the findings for a linkage between hypertension and a locus on chromosome 17.

1. Introduction

Hypertension is the most common cardiovascular disease. According to the Spanish Society of Hypertension [1] 35% of the general adult population in Spain, of whom 40% are in middle age and 60% beyond the age of 60 years, have hypertension. A large majority of hypertensive patients need long-term administration of antihypertensive agents and, therefore, patients are exposed to prolonged contact with these drugs. In prescribing a drug the knowledge of the benefit/risk ratio is of fundamental importance. For antihypertensive drugs the long duration of the pharmacological treatment of patients requires documentation of long-term safety and efficacy, including sensitive indices of genotoxic damage.

A recent review about genotoxicity and carcinogenicity studies of antihypertensive agents [2] suggests that for many antihypertensive drugs the published data may not allow the evaluation of the genotoxic and carcinogenic risks to humans. The review [2] reported the toxicological data available for each of 11 classes of antihypertensive agents. Only in 99 of the 164 marketed antihypertensive drugs there was at least one result from genotoxicity or carcinogenicity assays and in just 34 of these 164 drugs there was at least one genotoxicity result in human cells.

Among the pharmacological class of β-blockers, results of genotoxicity studies were retrieved for 23 of the 35 marketed β-blockers. For one of these β-blockers, atenolol, there are only two studies in humans, one in primary hepatocytes [3] and our previous work [4] in peripheral lymphocytes.

The genotoxicity of atenolol was cytogenetically tested by assessing its ability to induce sister chromatid exchange (SCE) and micronuclei (MN) in cultured peripheral lymphocytes of treated patients and control individuals [4]. The frequency of SCE did not show significant differences. However, a statistically significant increase in the frequency of MN was detected in treated patients. Application of the fluorescence *in situ* hybridization (FISH) technique with an alphoid satellite probe revealed a statistically significantly higher percentage of centromere-positive MN in this group, indicating the more frequent involvement of aneuploidy (specifically chromosome loss) in the origin of atenolol-induced MN *in vivo*.

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The fundamental question from which chromosome and chromosome loci the MN are derived, remains to be answered. Previous studies have found a non-random distribution in the observed number of structural chromosomal aberrations (CAs) [5,6]. Also, a non-random distribution was found in studies of spontaneous chromosome loss [7,8]. These results indicate that non-random processes may underlie the formation of CA (gaps and breaks) and the chromosome fragments that give rise to MN [9–15]. The review by Norppa and Falck [16] also reported that there are clear indications that different chromosomes are micronucleated non-randomly.

The view that a non-random process is operating in the formation of atenolol-induced MN *in vivo* was examined in the study reported here. The following two general approaches were chosen.

1.1. Analysis of fragile sites (FS)

Chromosomal fragile sites (FS) are specific loci that preferentially exhibit gaps and breaks on metaphase chromosomes. The first fragile site on a chromosome was described in 1965 by Dekaban [17]. In 1968 Lejeune et al. [18] considered them as heritable chromosomal markers. However the term "Fragile Site" was not introduced until 1970 by Margulis et al. [19]. There are two criteria for their classification: the prevalence and the mode of induction *in vitro*. According to the first criterion, they are classified as common fragile sites (cFS) when they can be observed in virtually every individual, and as rare fragile sites (rFS) when they are observed in relatively small percentage of the population [20]. According to the second criterion there are several groups, based on the culture conditions in which they are expressed [21].

Both rare and common FS are involved in chromosomal instability. Following induction in tissue culture, fragile sites were found to be preferably involved in SCE, deletions, translocations, intrachromosomal gene amplification and integration of foreign DNA. Through these chromosomal aberrations, FS may play a role both in loss of tumour-suppressor genes and in amplification of oncogenes [22]. Also a connection between FS and the cellular DNA-damage response was demonstrated. Expression of FS is elevated in *Ataxia Telangiectasia* and Rad3-related (ATR) deficient cells and in cells from patients with Seckel syndrome, which carry a mutation in the *ATR* gene. ATR is a major component of the DNA-damage response pathways in the cell [23].

It has also been proposed that FS may be preferential targets for mutagens and carcinogens [24] and that they represent regions of the genome that are particularly sensitive to genotoxic stress [25].

The information on FS summarized above indicates that non-random processes underlie the expression of FS. The analysis of the FS significantly expressed in our samples would allow the identification of possible specific and exclusive FS in hypertensive patients treated with atenolol.

1.2. Analysis of micronuclei (MN)

Micronuclei (MN) are functionally defined as small, round to oval bodies within the cytoplasm of a cell, having the same staining properties as the major nucleus, and assumed to arise from the loss of a fragment or a whole chromosome from the nucleus [26].

The application of the MN test in human lymphocytes was first explored by Countryman and Heddle in 1976 [27]. Until now this cytogenetic end-point has been used in many studies for the identification of genotoxic effects such as chromosome damage and aneuploidy associated with mutagenesis and carcinogenesis (see, *e.g.*, [16,28–31]).

Centromere identification has been successfully used in numerous *in vitro* and *in vivo* studies to examine the content of MN. Fluorescence *in situ* hybridization with DNA probes detected by use of fluorescence microscopy has been applied in most studies [16].

In contrast to the many studies concerning the presence of a centromere in a MN, only a few investigations have attempted to answer the question which chromosomes are involved in MN formation [32]. To our knowledge, there are no published data using this assay in humans to assess chromosomal damage of the β-blocker atenolol.

In this study we applied the *in vivo* MN assay. The origin of MN was determined by the FISH technique with a probe detecting all human centromeres, and by use of chromosome-specific probe(s) for the chromosome(s) with a significant level of expression in the previous analysis of FS, in order to assess whether that/those chromosome(s) is/are preferentially prone to MN formation.

2. Materials and methods

2.1. Sample

The study comprised two different groups: 11 hypertensive patients and 9 control individuals, matched with respect to sex, age and smoking habit (in this present study all individuals were non-smokers). Patients (eight male and four female) ranged in age from 39 to 79 years with an average of 59.3 years. All of them received a dose of 50 mg atenolol/day by oral administration, except for one patient receiving 25 mg atenolol/day and two patients receiving 100 mg atenolol/day. The average duration of treatment was 4.7 years (8 months–10 years). None of them was undergoing other medical treatments. They did not have a previous history of exposure to genotoxic compounds or a recent X-ray examination. Each patient was given a code number (P1–P11) according to the sequence of blood collection. Control individuals (six male and three female) had an average age of 54 years. All of them were healthy individuals not under any medication, with no alcohol or drug consumption in the recent past. Each control individual was given a code number (C1–C9) according to the sequence of arrival at the laboratory. Informed consent was obtained from all individuals to take part in the study.

The four hypertensive patients (P1–P4) and the four control individuals (C1–C4) analyzed in our previous study [4] were included in the present sample. Only data from the MN assay and FISH with centromeric probes in the mentioned individuals were included in that publication, whereas the data from these analyses in the other 12 individuals and data from CAs analysis, chromosomal location of CAs and FISH with chromosome-specific probes in all 20 individuals have not been published until now.

Samples from treated patients and control individuals were obtained between 1999 and 2003 and cytogenetic analyses started immediately. All the cytogenetic parameters were analyzed in the year following the sample arrival so samples were stored during 1 year at most. Analysis by means of FISH with chromosome-specific probes was not initially planned. The results obtained until then led us to include this analysis, so only in this case frozen samples for more than 1 year were analyzed.

2.2. Cytogenetic tests

Venous blood was taken from each subject and duplicate whole blood cultures were set up by adding 0.5 ml of heparinized blood to 4.5 ml of RPMI 1640 medium, supplemented with 10% foetal bovine serum, antibiotics (penicillin and streptomycin), glutamine and Hepes buffer solution. Lymphocytes were stimulated with 4% phytohaemagglutinin. All materials mentioned in this paragraph were obtained from Gibco.

For the FS analysis, the cultures were incubated at 37 °C for 72 h. One hour prior to harvest, 0.4 μg/ml of colcemid (Gibco) was added to arrest the cells at metaphase. For the MN assay, the cultures were also incubated at 37 °C for 72 h. BN cells were accumulated by adding cytochalasin-B (Cyt-B) (Sigma) at a final concentration of 6 μg/ml [33] at 48 h following initiation of culture. At the end of the incubation time, the cells were collected by centrifugation and, for FS analysis, resuspended in a pre-warmed hypotonic solution (0.075 M KCl) for 10 min and fixed three times in methanol:acetic acid (3:1). Cells were spread on slides, air-dried and stained with Giemsa. For the MN assay, the cells were washed once in RPMI 1640 medium and then a mild hypotonic treatment (2–3 min in 0.075 M KCl at room temperature) was carried out. The cells were then centrifuged and a methanol:acetic acid (5:1) solution was added. This fixation step was repeated twice. Air-dried preparations were made and the slides were stained with 10% Giemsa in phosphate buffer for 20 min.

2.3. Fluorescence in situ hybridization (FISH)

For the identification of centromeres in MN, FISH was performed with a probe for all human centromeres (Q-BIOgene, PAHC0001-G) following the protocol recommended by the manufacturer. Slides were incubated for 30 min at 37 °C in 2× SSC/0.5% NP-40, pH 7.0 and then dehydrated in a series of ice-cold ethanol