High blood pressure related to carcinogen-induced unscheduled DNA synthesis, DNA carcinogen binding, and chromosomal aberrations in human lymphocytes

(aging/DNA repair/essential hypertension/N-acetoxy-2-acetylaminofluorene/7,12-dimethylbenz[a]anthracene)

RONALD W. PERO*, CARL BRYNGELLSON*, FELIX MITELMAN††, THOMAS THULIN†, AND ÅKE NORDÉN†

* Department of Biochemistry, Nucleic Acids Research Group, University of Lund, Box 740, 220 07 Lund, Sweden; † Unit of Community Care Sciences, 240 10 Dalby, Sweden; and † Department of Clinical Genetics, University Hospital, 221 85 Lund, Sweden

Communicated by Åke Gustafsson, April 20, 1976

ABSTRACT Unscheduled DNA synthesis (excision-repair) of N-acetoxy-2-acetylaminofluorene (NA-AAF) damage to the DNA of human lymphocytes was determined quantitatively for 92 individuals with diastolic blood pressures ranging from 65 to 120 mm of Hg (8.7 to 16 kPa). Measurements of NA-AAF-induced repair synthesis (incorporation of [3H]thymidine in the presence of 10 mM hydroxyurea) showed linear increase with the blood pressure in the individuals under study. Concurrent determinations for the levels of [3H]-labeled 7,12-dimethylbenz[a]anthracene bound to the DNAs of lymphocytes after 18 hr of culturing have shown that increased amounts of DNA-bound carcinogen were linearly proportional to increased NA-AAF-induced repair synthesis values, and therefore were correlated to high blood pressure. The number of NA-AAF-induced chromosomal aberrations in lymphocytes increased linearly with the diastolic blood pressures of the individuals. High NA-AAF-induced repair synthesis values also tended to be associated with increased NA-AAF-induced chromosomal damage. Together, these results suggest that individuals with elevated blood pressures have a greater potential for accumulating DNA damage, because of an increased chemical reactivity of lymphocytes to carcinogen exposure, than do individuals with normal blood pressure.

The importance of high blood pressure as a major factor in cardiovascular disease is well established (1–3), and recently it has also been correlated to increased cancer risk (4). These clinical observations have suggested to us the possible involvement of genetic mechanisms which might regulate the intrinsic mutational frequency of blood cells (5). Therefore, our laboratory has sought to define quantitatively at least one potential altering mechanism of genetic information; namely, the level of DNA repair synthesis in relation to an individual's blood pressure.

The demonstration that human cell types undergo unscheduled DNA synthesis (excision-repair) following appropriate stimulation by DNA-damaging agents is now well documented (6–10). This observation suggests that accumulation of genetic damage in humans must be, in part, dependent on the efficiency with which the repair enzymes remove any environmentally induced chemical alterations within the genome. This is apparently true in at least some cases, since defective repair of damaged DNA has been correlated with carcinogenesis in the skin disease xeroderma pigmentosum (11) and in ethyl nitrosourea carcinogenesis in the rat (12). Moreover, the capacity for repair of genetic damage has been linked in vitro to the ageing process in human fibroblasts (13), as well as correlated to the lifespans of several mammalian species (14). However, this connection to age is not quite clear, since repair of UV- and x-irradiation damage of human fibroblasts has also been shown to correspond only slightly or not at all to the lifetime of the cells in culture (15–17). Nonetheless, several other studies (18–20) have related the accumulation of chromosomal aberrations with an increase in age of the animal. The physiological age, rather than the chronological age, of the donor was most important in shortening the lifespan of cultured human fibroblasts (21). Since high blood pressure is a physiological state associated with a reduced life expectancy, it was thought that a rise in blood pressure might signal an accelerated ageing process when compared to chronological age itself. Consistent with this reasoning, we have examined DNA repair synthesis, carcinogen binding, and chromosomal aberrations in human lymphocytes to determine if individuals with high blood pressure have the potential to accumulate abnormal levels of DNA damage.

METHODS AND MATERIALS

Lymphocyte Samples. A population in Dalby, Sweden, which had been monitored for blood pressure over a 5-year period, was used to select 92 males for this study. There were 25 individuals who were selected for having slightly low or normal blood pressures. Another 26 individuals were selected for having slightly higher than normal blood pressure for their age. The remaining sample was composed of 41 patients with diagnosed essential hypertension. A total of 51 patients were receiving treatment for high blood pressure at the time of sampling. Just prior to the taking of blood, the systolic and diastolic blood pressure was measured in the right arm for each individual following 10–15 min of rest in a horizontal position. Approximately 50–70 ml heparinized peripheral blood was collected and the lymphocytes were isolated within 1–2 hr after sampling. The blood samples were diluted with an equal volume of physiologic saline and 30 ml portions were carefully layered on top of 20 ml sodium metrizoate-Ficoll solution (1.077 g/ml, Lymphoprep, Nyegaard and Co.). Lymphocytes were collected from the interphase zone after centrifugation at 400 X g for 35 min. The cells were washed with physiologic saline and resuspended in Eagle’s medium fortified with 20% fetal calf serum, 2.5 mM L-glutamine, penicillin (50 µg/ml), and streptomycin (50 µg/ml). The cells were counted in a hemocytometer and adjusted to 2 × 10⁶ cells per ml of culture medium. All incubations of lymphocyte cultures were carried out at 37°C.

Measurement of Repair Synthesis. The carcinogen N-acetoxy-2-acetylaminofluorene (NA-AAF) was supplied by the Frederick Cancer Research Center (National Cancer Institute, USA). Thermal elution chromatography, S₁ nuclease-digestion studies, and pyrimidine isostich analyses with NA-AAF-induced unscheduled DNA synthesis have shown that there is an
accurate restoration of the damaged area to the original nucleotide sequence (29, 33). The NA-AAF was dissolved in di-methyl sulfoxide (Me2SO) and added to 10 ml of lymphocyte cultures (about 20 × 10^6 cells), so that upon dilution the desired NA-AAF concentration was reached without exceeding a final Me2SO concentration of 0.75%. Similar lymphocyte cultures containing only the corresponding amounts of Me2SO served as controls. The NA-AAF-treated and Me2SO cultures were incubated 1 hr before the lymphocytes were sedimented, washed with saline, and resuspended in fresh medium at 2 × 10^6 cells per ml. Next, 10 mM hydroxyurea and [3H]dThd (10 μCi/ml, 21 Ci/mmol, Amersham) were added and the cells were incubated 17 hr longer. It has been shown previously (6, 24, 25) that hydroxyurea suppresses [3H]dThd incorporation due to replicative synthesis without seriously affecting repair synthesis. The lymphocytes were harvested by centrifugation and the DNA was extracted by a modification of the Marmur method (26). Solutions of 1 M Tris and 5 M NaClO4 were added to the DNA extract so that, after addition of an equal volume of 0.4 M phosphate buffer (equimolar mixtures of NaH2PO4 and Na2HPO4), the final concentrations were 0.17 M and 0.85 M, respectively. The resultant DNA extract was then shaken with an equal volume of CHCl3-isooamyl alcohol (24:1, vol/vol) and the aqueous phase was mixed directly with 1.0 g of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) at room temperature. The slurry was mixed for 20 min, and the hydroxyapatite was separated by centrifugation and then washed again with 0.2 M phosphate buffer in 3 M KCl. The DNA was removed from the hydroxyapatite in 0.4 M phosphate buffer, precipitated with 7% trichloroacetic acid, immobilized onto nitrocellulose filters, and washed with 70% (vol/vol) ethanol. Approximately 20 μg of DNA was immobilized onto each nitrocellulose filter. The dried filters were counted in a toluene-based scintillation mixture, washed free from scintillation liquid with CHCl3 after counting, and the quantity of DNA was determined by a modified diphenylamine procedure (27). Background replicative synthesis was estimated by the dpm of [3H]dThd per μg of DNA present in the hydroxyurea plus Me2SO controls. The influence of background replicative synthesis has been subtracted from all the NA-AAF–induced repair values.

Measurement of Carcinogen Binding. In some cases, along with the measurements of NA-AAF–induced repair, parallel lymphocyte cultures were treated with 7,12-dimethylbenz[a]-anthracene (DMBA) dissolved in Me2SO to measure carcinogen binding to the DNA. This carcinogen was considered a good model for DNA binding studies because (a) the binding indexes for DMBA and related polynuclear hydrocarbons (28) have been shown to correlate well to their carcinogenicity and (b) DMBA probably needs metabolic activation for covalent binding to DNA (29) and, therefore, would also reflect any biologic differences in lymphocyte metabolism associated with carcinogen uptake and reactivity. For this purpose, the lymphocyte cultures were exposed to 5 μM [3H]DMBA (7 Ci/mmol, Amersham) for 18 hr before the cells were harvested by centrifugation, the [3H]DMBA-containing medium was decanted, and the cells were resuspended and pelleted again in physiologic saline to remove any traces of exogenous [3H]DMBA. The DNA was extracted, precipitated with 7% trichloroacetic acid, and loaded onto filters as described above. The filters were then washed for 1 hr with gentle stirring in petroleum ether (80°–110°) to remove any loosely bound [3H]DMBA. The washed filters were counted, the amount of DNA was determined, and the results were recorded as dpm of [3H]DMBA per μg of DNA.

RESULTS

Before carcinogen-induced repair synthesis can be evaluated quantitatively, it must be shown that the values that are calculated accurately reflect the amount of repaired DNA damage. This was done for NA-AAF–stimulated repair by correlating the DNA damage induced by increasing concentrations of NA-AAF in the culture medium to increasing levels of [3H]dThd incorporation due to repair synthesis. It is clear from Fig. 1 that there is a linear relationship between [3H]dThd incorporation due to repair and the concentration of NA-AAF to which the lymphocytes were exposed. Linearity was lost above 15 μM NA-AAF, indicating a lack of quantitation at the higher doses of the carcinogen. Therefore, we have selected 10
μM NA-AAF as optimum to quantify our repair synthesis measurements, since at this concentration the [3H]dThd incorporation was presumably directly proportional to the number of damaged sites in the DNA.

The reproducibility of NA-AAF repair values was calculated for the lymphocytes from the same individual by repeating determinations in the same experiment and by repeating the experiment at different time intervals. Table 1 shows that the method is reproducible to ±9.0% of the mean value in a single experiment wherein mainly physical and chemical variables in the method are taken into consideration. However, when biological variation is also considered, as is the case when replicating experiments, the variation from the mean value is increased to ±15.7%. These data indicate that NA-AAF-repair values are repeatable for an individual, but, because of the high level of observed biological variation, the method is limited to large sampled populations for appropriate statistical analyses.

The data relating blood pressure to repair synthesis are presented in Fig. 2. When the NA-AAF repair values for the entire sampled population were compared to diastolic blood pressure values, both parameters increased proportionally to each other in a linear fashion (Fig. 2A). This was true even though the sampled group contained "normal" individuals with low or elevated blood pressures as well as patients with hypertension, whether treated or not. A similar correlation to systolic blood pressure was also observed, and a multivariate analysis showed the correlation between blood pressure and repair to be independent of age effects (data not shown). The linear relationship between NA-AAF repair and diastolic blood pressure was not dependent on the treatment some of the patients were receiving for high blood pressure, since the data in Fig. 2B show that non-treated individuals had the same correlation to repair as the entire sampled population did. As might have been predicted from the clinical evidence that blood pressure rises in a population as does age (2, 3), the amount of NA-AAF repair not only increased with elevated blood pressure, but also increased significantly with age (Fig. 2C). This was true even though the sampled population was biased to select individuals with high blood pressure. Statistical analysis showed the increase in NA-AAF-repair was highly significant whether the ≥61-yr-old age group was included in the analysis or not. However, when the 51- to 60- and ≥61-yr-old age groups were compared directly, a tendency toward a decline was observed (analysis of variance, P < 0.10). The reason for this tendency is unclear, but a similar peak in the occurrence of hypertension among males also occurs around 50 years of age (2, 3).

The differences in NA-AAF repair between various blood-pressure and age groups might be explained simply by differential [3H]dThd uptake or by different numbers of lymphocytes in S phase which escape the hydroxyurea replicative synthesis block at the time of sampling. However, this does not seem to be the case, since the [3H]dThd uptake in the presence of hydroxyurea did not vary significantly between the different blood pressure and age groups measured in Fig. 2.

Chemically induced DNA damage, as opposed to x- or UV-irradiation damage, is directly dependent on cellular transport mechanisms for the agent to reach the site of reactivity with DNA. Any differences in lymphocyte metabolism from patients with different blood pressures that might affect either carcinogen uptake or DNA binding would greatly influence the potential for accumulation of genetic damage. The data in Fig. 3A provide the necessary basis for quantifying DMBA binding to lymphocyte DNA. As the concentration of DMBA was increased in the lymphocyte culture medium, the level of DMBA.

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Individual values</th>
<th>Mean</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyzed as replicates in 1 experiment</td>
<td>719, 691, 724, 689, 532, 677, 701</td>
<td>676</td>
<td>9.0</td>
</tr>
<tr>
<td>0–9 weeks, 8 experiments</td>
<td>523, 542, 379, 427, 626, 456, 676, 556, 523</td>
<td>523</td>
<td>15.7</td>
</tr>
</tbody>
</table>

All the repair values were calculated on the same individual after exposure to 10 μM NA-AAF according to the protocol in Methods and Materials.
The levels of carcinogen binding in the lymphocytes from 25 individuals have been determined at a standard dose of 5 μM DMBA. Six individuals with diastolic blood pressures of 100–115 mm of Hg bound 160 ± 75 dpm of [3H]DMBA per μg of DNA, whereas 19 individuals with diastolic blood pressures of 70–95 mm of Hg bound 108 ± 36 dpm of [3H]DMBA per μg of DNA (P < 0.05). Fig. 3B displays the same DMBA binding data plotted against NA-AAF-induced repair values that were collected on the same individuals at the same time. There was a linear association between DMBA binding and NA-AAF-induced repair values, suggesting that DMBA binding to lymphocyte DNA also increases linearly with blood pressure, since a similar correlation to NA-AAF-induced repair has already been shown (Fig. 2). This observation would be significant whether or not the correlation between DMBA binding and NA-AAF-induced repair was biological or mathematical in nature.

Cytogenetic analyses of NA-AAF-damaged lymphocytes have confirmed our biochemical observations. The percent of chromosomal aberrations, which were characterized mainly as chromatid breaks, increased in linear relation to increases in the diastolic blood pressure for the 19 individuals examined (Fig. 4A). Furthermore, when biochemical determinations of NA-AAF-repair synthesis were carried out simultaneously in the same individuals that cytogenetic evaluations were made in, the percent chromatid breaks tended to increase as did the NA-AAF-repair values (Fig. 4B). With a small sample size of 13, the results were not statistically significant (P < 0.20).

Together, the biochemical and cytogenetic evidence in this study supports the interpretation that there is a greater potential for accumulation of DNA damage in lymphocytes from high-blood-pressure individuals, because their lymphocytes have higher sensitivity to carcinogen damage than do lymphocytes from normal-blood-pressure individuals.

**DISCUSSION**

The level of excision-repair synthesis is directly dependent on the initial amount of DNA damage, since it is the damaged sites...
in DNA that provide substrate for driving the enzymatic repair reactions. Our DMBA binding and cytogenetic data (Figs. 3 and 4) have provided evidence that carcinogen-treated lymphocytes from high-blood-pressure individuals have larger initial amounts of DNA damage than do lymphocytes from normal-blood-pressure individuals. If this is true, then quantification of repair synthesis in lymphocytes from individuals with different blood pressures only reflects the different degrees of repair stimulation coming from the different levels of initial DNA damage. In fact, that is what our data indicate, because high ,45-90,44-90 high-blood-pressure damage, stimulation coming from potential techniques. It is tempting to view our results in terms of the mutational model for cancer and ageing (5, 30–32). Presumably the increased potential for DNA damage observed within the lymphocytes of high-blood-pressure individuals would be expressed through successful mutational hits as an increased potential for genetic damage. The role of genetically altered lymphocytes in ageing, cancer, and hypertension is unknown and involves even greater speculation. Nonetheless, impairment of T-cell immune responses in ageing (35) and the relation of immunosuppression to cancer (34) have indicated the importance of lymphocytes as target cells during these disease processes.

It is also of interest to compare the lifespans of mammals to the recorded blood pressures that are available. Mammals such as the chimpanzee, horse, and human with long lifespans of 44–90 years have an average diastolic blood pressure of 76 ± 7.9 mm of Hg, but 15 different mammals with shortened lifespans of 3.5–35 years had an average diastolic blood pressure of 97 ± 13.3 mm of Hg (35, 36). Of course, different measurement techniques for blood pressure were used and in some cases only a few animals were examined. Such important limitations do not permit any valid conclusions. Therefore, this observation may be irrelevant, but it does serve to direct attention to the possible importance of blood pressure in ageing.

This study was supported by a special grant to the Dalby Community Care Sciences program from the National Board of Health and Social Welfare in Sweden. We are indebted to Drs. Adam Deutsch and Bengt Scherstén for their expert advice on this study.