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CHROMATIN AND DNA REPAIR AFTER TREATMENT WITH SIMPLE ALKYLATING AGENTS; RELATION TO CARCINOGENESIS.

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Methyl- and ethyl-nitrosoureas, dimethyl- and diethyl-nitrosamines may be taken as examples of simple alkylating agents which are carcinogenic. The alkylnitrosoureas are direct carcinogens : they react with DNA in vitro. The dialkylnitrosamines are indirect carcinogens : they must be activated metabolically to yield the ultimate carcinogens which react with DNA. These simple carcinogens alkylate the nitrogens and the oxygens in DNA.

The alkylated nitrogens are mostly the N-7 of guanines and N-3 of adenines. The alkylation brings a positive charge whose delocalization is responsible for the loss of the alkylated purine by hydrolysis of the glycosyl bond with the deoxyribose of the polynucleotide backbone. Moreover, cells contain enzymes, called DNA glycosylases, which catalyze the hydrolysis of the same glycosyl bonds, accelerating the loss of the alkylated purines. The departure of the purine leaves in DNA an apurinic site or AP site. AP sites are very common lesions in DNA exposed to simple alkylating agents.

Oxygen alkylation in DNA occurs on phosphates, O-6 of guanines, and pyrimidines. A good correlation exists between the carcinogenic activity of a simple alkylating agent and the alkylation of oxygens. Alkylation of guanine O-6 seems particularly important, but the relevance of the alkylation of pyrimidine oxygens has not yet been sufficiently assessed.

Several theories try to explain the carcinogenic action of O^6-alkylguanine. O^6-Alkylguanine can pair with cytosine, but it can also mispair with thymine and be mutagenic; one rationalizes that a mutation in the promoter region might

awake an oncogene. Another theory is that the target guanine belongs to a methylated CG pair and that alkylation of guanine 0-6 prevents methylation of cytosine in the new strand when DNA replicates; methylation is completely lost after two cycles of DNA replication and undermethylation of the promoter might awake an oncogene.

The fate of these theories or any others to explain how alkylation of guanine 0-6 leads to cancer is of no consequence for the present discussion, the only important point is that disappearance of O^6-alkylguanine before DNA replicates seems to prevent malignant transformation. DNA does not loose spontaneously O^6-alkylguanine, but, in vivo, O^6-methyl- and O^6-ethylguanine disappear from the DNA of many cells. If alkylation of guanine 0-6 is the principal cause of cancer induced by simple alkylating agents, the probability of a cell malignant transformation will, in principle, depend on several parameters : the level of guanine O^6-alkylation of the cell DNA; the activity of the repair system which removes the potentially carcinogenic lesion; the delay before the cell DNA replicates since, to be useful, the repair must be completed before the replication fork passes over the modified base.

This paper deals with the repair of two major lesions that appear in DNA of cells exposed to simple alkylating agents : AP sites and O^6-alkylguanine.

REPAIR OF AP SITES :

Enzymes that recognize specifically AP sites in DNA and nick the damaged strand near the lesions have been found in bacterial, animal and plant cells (Verly et al. 1973). These enzymes are called AP endodeoxyribonucleases (or simply AP endonucleases).

Thibodeau and Verly (1980) have studied the cellular localization of the AP endonucleases in rat liver. The apparent activity tested on an added DNA containing AP sites is mostly located in the nucleus, although some activity is found in the cytoplasm. The cytoplasmic activity is shared between mitochondria, membranes and cytosol. In the nucleus, most of the apparent activity is in the nuclear sap and some in nuclear membranes and native chromatin. However, if chromatin is dissociated with heparin-Sepharose and the complex extracted with 0.5 M KCl, the activity of the non-histone protein solution is 10 to 50 times higher than the apparent

activity of the undissociated chromatin (César and Verly 1982). The final conclusion is that more than 90% of the cell AP endonuclease is in chromatin; in native chromatin, this activity is masked in the sense that it is unable to work on an added foreign DNA perhaps because the enzyme, firmly integrated in the chromatin structure, is oriented to scan the chromatin DNA. Partial digestion with micrococcal nuclease has shown that the chromatin AP endonuclease is present in the cores as well as in the linkers of the nucleosomes (Bricteux and Verly 1982).

The chromatin AP endonuclease has been purified. Two isozymes are separated on hydroxyapatite chromatography : one is eluted with 0.2 M phosphate, the other with 0.3 M phosphate. The 0.2 M/0.3 M isozyme ratio in chromatin extracts depends on the method of preparation; when protease inhibitors are used at all steps and the time to the end of the hydroxyapatite chromatography is kept at a minimum, the proportion of the 0.2 M isozyme can be lower than 5% (Bricteux et al 1982). The 0.2 M isozyme is thus mostly an artifact and it seems that the 0.3 M species is the true chromatin enzyme. This enzyme has been completely purified (César 1982); it has a molecular weight of 42,000; it hydrolyzes the phosphodiester bridge 5' to the AP sites leaving 3'-hydroxyl and 5'-phosphate ends (César and Verly 1982).

The nuclear sap enzyme has not yet been completely purified. It is eluted from hydroxyapatite with 0.2 M phosphate so that it is different from the main chromatin AP endonuclease. Cytosol and nuclear sap enzymes have the same physical properties so that they are probably the same protein, but the AP endonuclease is 200 times more concentrated in the nuclear sap than in the cytoplasm.

The membrane AP endonuclease is different from the chromatin and nuclear sap enzymes; it is an intrinsic membrane protein that cannot be removed by repeated washings with 0.25 M NaCl. Electron microscopic examination of microsomes prepared from rat liver shows the ribosomes attached on the outside of the vesicles; since these vesicles are active on an added DNA containing AP sites, the membrane AP endonuclease is likely located on the cytoplasmic side of the endoplasmic reticulum.

To summarize, rat liver contains several different AP endonucleases and each one is located in a particular cell compartment. It is likely that the only AP endonuclease used for the repair of nuclear DNA in the living cell is the

chromatin enzyme. Thibodeau and Verly (1980) have speculated that the AP endonucleases in other cell compartments might be precursors of the chromatin enzyme. To take account of all the experimental data, they proposed the following model the precursor is synthesized on free ribosomes and, because of a hydrophobic end, attaches to the cytoplasmic side of the endoplasmic reticulum; this membrane enzyme is carried in the nucleus as on a conveyor belt by the membrane system; a protease, cutting away the hydrophobic end, releases the nuclear sap enzyme; very little nuclear sap enzyme leaks into the cytoplasm, most of it is modified to go into the chromatin. It is possible that this model is not unique for the AP endonuclease and that nuclear DNA repair enzymes are generally synthesized as precursors. This hypothesis is relevant to oncology since lack of maturation of these enzyme precursors would cause DNA repair deficiencies with their increased probability of cancer.

Goffin and Verly (1982) have repaired in vitro DNA containing AP sites with enzymes extracted from rat liver chromatin : the AP endonuclease nicks the damaged strand 5' to the AP site; a 5'-3' exonuclease excises the AP site in an oligonucleotide (Zocchi 1982); DNA polymerase fills the gap and ligase closes the last break. It is not however because all the enzymes necessary to repair naked DNA containing AP sites are found in chromatin, that the in vitro model represents what happens in vivo; it is probably more complicated in the living cell because of the complex structure of chromatin. Repair of DNA in reconstituted chromatin should be explored.

REPAIR OF O^6-ALKYLGUANINE :

Rat liver has a repair activity responsible for the disappearance, from DNA, of O^6-methylguanine and O^6-ethylguanine which are potentially carcinogenic. The cellular localization of this activity again shows that most of it is in chromatin; some is also found in nuclear sap and cytoplasm (Renard and Verly 1980, 1982). It is not yet known whether the cytoplasmic and nuclear sap enzymes are different from the chromatin enzyme. All the results reported hereafter were obtained with the chromatin enzyme.

The preparation of chromatin enzyme is obtained in the following way : cell nuclei are prepared from rat liver; chromatin is prepared from the purified nuclei and dissociated

with heparin-Sepharose; the complex is eluted with 0.3 M KCl to get the chromatin protein solution which is dialyzed against a suitable buffer. The substrate is prepared by alkylating DNA either with (H^3-methyl)nitrosourea or with (H^3-ethyl)nitrosourea. The alkylated DNA is incubated with the chromatin proteins and, at the end of the incubation, DNA and protein are separated by isopycnic centrifugation in CsCl gradients. In the non-incubated controls, all the radioactivity is in the DNA band; after incubation, part of the radioactivity is found in the protein band. The same observation is made with ethylated DNA (Mehta et al. 1981) and methylated DNA (Lemaître et al. 1982). Protein and DNA are subsequently analyzed separately; the example of the repair of ethylated DNA will be followed.

The DNA is enzymatically hydrolyzed to nucleosides which are separated by HPLC. In the non-incubated control, there is a peak of O^6-ethyldeoxyguanosine. This peak has nearly disappeared after incubation with the chromatin proteins; the other peaks have barely changed and no new peak has appeared (Mehta et al. 1981).

A radioactivity equivalent to that lost by DNA as O^6-ethylguanine is found in the protein fraction. Trypsin digestion followed by HPLC analysis gives two radioactive peaks. Hydrolysis of these oligopeptides with pronase and aminopeptidase yields radioactive S-ethylcystein (Mehta et al. 1981). The repair is thus a transethylation from O^6-ethylguanine in DNA onto two cysteins of acceptor proteins. Similar results are obtained when methylated DNA is repaired (Mehta et al. 1982).

The reaction rate is faster with O^6-methylguanine than with O^6-ethylguanine, but the same limiting component (likely the acceptor protein) is used up in the repair of both lesions : when this limiting component is exhausted by a preliminary incubation with methylated DNA, no repair of O^6-ethylguanine lesions can be observed when ethylated DNA is added, and vice versa (Lemaître et al. 1982).

The repair is not a simple one-step reaction between O^6-ethylguanine in DNA and the cystein of the acceptor protein; such a bimolecular reaction should depend on a second order kinetic constant which is not found. At least a two-step reaction must be considered in which the overall reaction is catalyzed by a transalkylase. It is still a matter of debate whether, in mammalian cell chromatin, the transalkylase and acceptor functions belong to the same or

different polypeptides (Renard et al. 1982).

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