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ENVIRONMENTAL EFFECTS CAUSING DNA DAMAGES

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ÖSSZEFOGLALÁS

DNS KÁROSODÁST ELŐIDÉZŐ KÖRNYEZETI HATÁSOK

Áttörést értünk el a sejt szinkronizálás terén, a DNS replikáció és repair egyidejű mérésével, permeábilis sejteken toxikus hatások mérése terén és azzal, hogy olyan kromatin szerkezeteket izoláltunk, melyekben a kromatin kondenzálást hosszú oldalláncú nukleotidok beépítésével gátoltuk.

A toxikus stresszt a DNS információ átvitel szintjén vizsgáljuk. Toxikus hatásra a replikatív DNS szintézis átmenetileg gátlást szenved, a repair szintézis pedig emelkedik. A két típusú DNS szintézist egyidejűleg követtük toxikus kezelés (nehéz fém, sugárzás) után és a DNS szintézisek profiljának lefutása ellentétes tendenciát mutatott. A toxikus hatás indikátoraként a DNS relikáció/repair arány szolgál. Számos replikációs ellenőrző pontot különböztettünk meg C-értéküк alapján és a Cd gátlás helyét is meghatároztuk. A replikációs profil felvétele a naszcens DNA közvetlen mérésén alapul. A toxikus hatások által okozott változásokat flow citométerrel regisztráltuk. Hasonlóképpen a repair szintézis ingadozása is közvetlenül detektálható a nagy tömegű celluláris DNS mérése nélkül.

SUMMARY

A recent breakthrough was achieved by our team with increasing the resolution of synchronization, by measuring simultaneously replicative and repair DNA synthesis and by using reversibly permeable cells for measuring toxic effects and isolating nuclear structures in which chromatin condensation has been hampered by the incorporation of nucleotides with long side chains.

We measure toxic stress at the DNA level in the transfer of genetic information. Toxic stress increases the rate of mutation resulting in changes in DNA synthesis. Upon toxic effect the replicative DNA synthesis is temporarily blocked, and the repair DNA synthesis is increased. The two types of DNA synthesis have been followed simultaneously upon toxic treatment (heavy metal, irradiation) and the opposite trends of DNA synthesis have been compared. The replicative /repair ratio of DNA synthesis has been used as a toxic indicator. Among the several replication control points distinction has been made based on the C-value of inhibition upon Cd treatment. We use nascent (newly replicated) DNA for the direct measurement of the replication profile and changes upon toxic effects by flow cytometry. Similarly repair patches can be followed by tracing exclusively repair DNA synthesis without measuring the bulky cellular DNA.

In the poster session (Szepessy, E. et al., Nagy, G. et al) of this volume we distinguish among intermediates of the interphase chromatin condensation-decondensation process. Toxic agents disturb the normal process of chromatin folding and result in chraracteristic changes in chromatin structure visualized by fluorescent microscopy. Repair and replicative DNA

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synthesis were measured at different stages of the cell cycle in control and in cadmium treated Chinese hamster ovary (CHO-K1) cells. Cells were synchronized by counterflow centrifugal elutriation. Increased resolution of elutriation resulted in five repair and four replication subphases. Upon Cd treatment repair synthesis was elevated in certain subphases. Multiple replicative subphases were suppressed by Cd treatment with some of the peaks almost invisible. The number of spontaneous strand breaks measured by random oligonucleoitede primed synthesis (ROPS) assay showed a cell cycle dependent fluctuation in control cells and was highly increased after Cd treatment throughout the S phase. Elevated level of the oxidative DNA damage product, 8-oxodeoxiguanosine was observed after Cd treatment with the highest level in early S phase which gradually declined as damaged cells progressed through the cell cycle.

INTRODUCTION

The incidence of cancer increases with age, with the increase of basal metabolic rate and with oxidative damage to DNA [1,2]. Some of the oxidative DNA damages are caused by reactive oxygen species such [3-8] do not react directly with DNA [9-11], while the ·OH radical generated by metal catalysis is an effective agent [6,7,10-12]. Heavy metals generate oxidizing radicals through Fenton chemistry and by the Haber-Weiss reaction leading to the hypothesis that metal carcinogenesis is mediated primarily by the elevated level of free radicals, reviewed by Kasprzak [13]. According to this view heavy metal induced oxidative stress can lead to different types of DNA damages as a consequence of consumption of molecular oxygen in multiple steps of incomplete O_2 reduction ultimately producing water.
Carcinogens regularly cause more than one type of modification. DNA damage suppresses DNA replication at checkpoints to avoid mutagenic changes to be perpetuated in the genome of the next generation of cells [14-16]. Our approach started with DNA synthesis in bacteria, ending up in eukaryotic cells [17-22]. The comparison of cell cycle profiles of replicative and repair synthesis in permeable cells showed opposite trends. The rates of repair synthesis and replication are inversely correlated [17].
A new approach has been taken for the *in vitro* detection of oxidative damage to DNA. The method is based on the measurement of replicative and repair DNA synthesis in permeable CHO cells. Changes in the maintainance of genetic information can be expressed by the ratio of replication/repair synthesis.

RESULTS

Effect of Cd concentration on replicative and epair ynthesis in exponentially growing cells
CHO cells were treated with $CdCl_2$ (0.2-1 mM) 9 hours after reculturing cells. Cells were grown for an additional 15 hours in the presence of $CdCl_2$ (Cd) before harvesting them. The observation that the biphasic replication profile comprising early and late S phases can be resolved into several subphases and the finding that repair and replicative DNA synthesis are inversely correlated [17,19] raised the question whether a DNA damaging agent causing mutations leading to carcinogenesis would change the profile of multiple subphases of the two DNA synthetic processes. To answer this question cadmium treatment of CHO cells has been chosen to induce oxidative stress leading to DNA damage. After Cd treatment the cells in logarithmic growth were subjected to counterflow centrifugal elutriation. Replicative and repair synthesis were carried out in synchronized populations of cells. DNA damage was followed based on the idea that major repair processes contain ATP-independent steps, the measurement of which represents repair synthesis [17].

Initially we have measured the effect of Cd concentration on repair and replicative DNA synthesis in exponentially growing cells. Increasing Cd concentration resulted in decreasing ATP-dependent replicative and elevated ATP-independent repair synthesis (Table 1).

Additions	pmol DNA synthesized		Ratio of replicative/repair synthesis
	ATP-dependent replicative synthesis	ATP-independent repair synthesis	
None	0.89	0.28	3.18
$CdCl_2$ 0.2 mM	0.67	0.47	1.43
0.5 mM	0.37	0.62	0.59
1.0 mM	0.32	1.33	0.24

Table 1. Effect of Cd concentration on replicative and repair synthesis in exponentially growing CHO cells

Replicative and repair DNA synthesis was carried out in 10^6 cells kept in culture for 24 hours. Cd treatment lasted for 15 hours before harvesting cells for DNA synthesis [29].
The inverse relationship is expressed by the ratio of replicative/repair synthesis changing from 3.2 in control cells to 0.26 in 1 mM Cd treated cells. This Cd concentration caused such a drastic drop in cell number that elutriation could not be carried out. For elutriation cells were treated with lower (0.5mM) Cd concentration. Even under these conditions drastic decrease in cell number was observed in Cd-treated group, nevertheless elutriation could be performed.

Replicative and repair synthesis in synhronized cells
The change in the rate of replicative and repair synthesis was followed throughout the S phase in synchronized populations of cells before and after 0.5 mM $CdCl_2$ treatment. For lower resolution of elutriation 8 fractions and for increased resolution 16 fractions were collected. Synchrony of fractions was confirmed by the simultaneous measurement of cell size in a Coulter Channelizer, by flow cytometry and by the determination of DNA content in each fraction. The comparison of the cell number in control and in Cd treated cells showed that 0.5 mM $CdCl_2$ prevented cell cycle progression and the cell number is lagging behind the control by more than 60%. Cd treatment shifted the growth profile of CHO cells toward the larger cell size. The shift in cell size was also visible under microscope. As a result of cellular damage after 0.5 mM $CdCl_2$ treatment only 10% high molecular weight DNA could be isolated from elutriated fractions relative to untreated control cells.
In elutriated fractions similar to unseparated cells the overall rate of DNA replication was decreased by more than 70 % in Cd treated cells with some of the smaller peaks almost invisible. The opposite tendency was observed in repair synthesis. Cd treatment increased the rate of repair synthesis. The overall rate of repair synthesis was 3 times higher in cells after Cd treatment.
A more detailed analysis shows the ratio of replicative and repair synthesis throughout the cell cycle in control and in cells treated with 0.5 mM $CdCl_2$. Tha ratio in elutriated fractions of control cells ranges between 0.7 and 11 with an average of 3.3 corresponding to unseparated cells (Table 2). The same analysis in cells damaged by Cd shows a reduced ratio between 0.02 and 2.4 with an average of 0.25 similarly to unseparated damaged cells (Table 3).

Elutriated fraction	Cell number ($x10^6$) (a)	pmol DNA synthesized Replication in 10^6 (b	Total (c= a . b)	Repair in 10^6 (d)	Total (e=a . d)	Ratio replication/repair (f=c/e)
Control	100	0.92	92	0.29	29	3.17
1	3.4	0.25	0.85	0.21	0.71	1.2
2	7.8	0.51	3.98	0.31	2.42	1.64
3	13.0	0.82	10.66	0.27	3.51	3.04
4	12.9	0.74	9.55	0.21	2.71	3.52
5	11.4	0.39	4.45	0.19	2.17	2.05
6	11.1	1.03	11.43	0.29	3.22	3.55
7	9.8	0.92	9.02	0.30	2.94	3.07
8	8.0	1.78	14.24	0.16	1.28	11.13
9	5.2	1.43	7.44	0.48	2.50	2.98
10	3.6	1.27	4.57	0.35	1.26	3.63
11	4.7	1.34	6.30	0.20	0.94	6.70
12	5.7	1.73	9.86	0.46	2.62	3.76
13	3.2	0.89	2.85	0.47	1.50	1.90
14	1.9	0.58	1.10	0.83	1.58	0.70
15	1.3	0.44	0.57	0.19	0.25	2.28
16	1.2	0.39	0.47	n.d	n.d	-
1-16 Total	104.2		97.3		29.61	3.29

Table 2. Ratio of replicative/repair DNA synthesis in synchronized populations of CHO cells

Exponentially growing cells were grown for 24 hours, harvested and 10^8 cells were subjected to elutriation. Replicative and repair synthesis in 106 cells were carried out as described in the Methods. n.d. = not detected [29].

Random primed oligonucleotide synthesis (ROPS assay)

Among the Cd2+ induced damages are single and double stranded breaks. By using the ROPS method [23] we could follow the cell cycle dependent variation of spontaneous strand breaks in control cells and after 0.5 mM $CdCl_2$ treatment. The level of spontaneous strand breaks indicates the number of free ends in control cells which is regarded as the reflection of short replication intermediates in oligonucleotide size range such as early replication intermediates [24], elongated intermediates of intermediate size such as Okazaki fragments and the accumulation of long fragments at the end of the S phase. After Cd treatment the number of strand breaks is increased by more than an order of magnitude 10 - 40 times greater than control throughout the cell cycle.. The relationship between strand breaks and DNA content could be estimated in control cells but not after Cd treatment due to the fact that DNA fragmentation caused by Cd reduced drastically the isolatable DNA. When the strand breaks of control cells were plotted against the total DNA content in a cell cycle dependent manner, highest percentage of strand breaks (22%) was found at the onset of S phase at 2.06 C value. C value represents haploid genom content.

Elutriated fraction	Cell number ($x10^6$)	pmol DNA synthesized Replication		Repair		Ratio of replication/ repair
		in 10^6	Total	in 10^6	Total	
	(a)	(b)	(c= a . b)	(d)	(e=a . d)	(f=c/e)
Unfractio-nated	100	0.35	35	1.33	133	0.26
1	0.9	n.d.	n.d.	n.d.	n.d.	-
2	2.5	0.14	0.35	0.25	0.63	0.56
3	3.8	0.17	0.65	0.21	0.80	0.81
4	5.1	0.11	0.56	0.25	1.78	0.31
5	5.4	0.17	0.92	0.46	2.48	0.37
6	6.0	0.20	1.20	9.19	55.14	0.02
7	5.7	0.27	1.54	0.45	2.56	0.60
8	4.4	1.09	4.80	0.46	2.02	2.38
9	3.5	0.24	0.84	0.50	1.75	0.48
10	3.4	0.13	0.44	0.39	1.33	0.33
11	2.6	0.23	0.60	0.51	1.33	0.45
12	4.5	0.41	1.84	0.76	3.42	0.54
13	4.6	0.24	1.10	1.78	8.19	0.13
14	4.1	0.90	3.69	0.62	2.54	1.45
15	5.3	0.31	1.64	0.72	3.82	0.43
16	15.4	0.26	4.00	0.90	13.86	0.29
1-16 Total	76.3		24.17		101.65	0.24

Table 3. Ratio of replicative/repair DNA synthesis in synchronized populations of CHO cells after Cadmium treatment

After renewal of culture cells were grown for 9 hours, then treated with 0.5 mM $CdCl_2$ for 15 hours. Cd treated cells were elutriated and DNA synthesis was carried out as described in the Methods [29]. n.d. = not detected

This percentage decreasing to about 10% at 2.7 C value, reaching a second peak (18%) in mid S phase at 3C and declining gradually to 7% by the end of S phase at 4C value. Taking into consideration the cell number in each fraction we have estimated the total number of strand breaks and found that there is a significant difference between Cd treated and control cells. The highest number of accumulating strand breaks was found in late S phase versus control in early S phase (results not shown).

Oxidative DNA damage

The presence of the oxidative DNA damage product, 8-OHdG was traced in elutriated fractions. The 8-OHdG level was low in control and was 30-60 times higher in cells treated with 0.5 mM $CdCl_2$. There was no significant change in the 8-OHdG content in early S phase and a gradual decrease to 50% was observed from the middle to the end of S phase (Table 4).

Number of elutriated fractions	8OHdG/10^5 dG
1	59.9 $\pm$ 12.5
2	54.7 $\pm$ 10.1
3	57.1 $\pm$ 14.8
4	38.8 $\pm$ 3.4
5	40.8 $\pm$ 3.8
6	23.5 $\pm$ 9.8
7	18.6 $\pm$ 4.2
8	25.9 $\pm$ 1.5
Control (1-8)	1-2

Table 4. Cell-cycle dependent 8-oxo-2'-deoxyguanosine content of CHO cells after Cd treatment

CHO-K1 logarithmic culture was harvested at 4 x 10^5/ml. $CdCl_2$ treatment (0.2 mM) for 15 hours. Counterflow centrifugal elutriation was carried out under lower resolution and 8 fractions were collected. Electrochemical detection of the intrinsic mutagenic indicator 8OHdG was by HPLC [29].

DISCUSSION

The role of reactive oxygen species in carcinogenesis is not completely understood at molecular level. Our study provides complementary information with respect to those processes which maintain the integrity of the genetic information, namely between DNA damage and repair as well as replicative and repair DNA synthesis. To strengthen the casual association between replicative and repair synthesis we have examined both processes side-by-side in a cell cycle dependent manner after Cd treatment of CHO cells. It is known that heavy metals induce cytotoxicity [25], that DNA strand breaks mediated by Ca-dependent endonucleases cause apoptosis [26] and oxygen radical species generated by heavy metal induced oxidative stress lead to carcinogenesis [27]. DNA damage caused by heavy metals such as Cd, Ni, Zn, Hg or Pb can be at least partially reversed by Mg [28]. The molecular mechanism of oxygen radical carcinogenesis is traced back to DNA base damages, the main products of which are 4,6-diamino-4-hydroxy-5-formamidopyrimidine ie. imidazole ring opened guanine and 8-hidroxyguanine, followed by the opening of the ring of adenine and cytosine [8].

Upon Cd treatment the enlargement of cells was visible under fluorescent microscope and could be measured in elutriated fractions by a Coulter Chanellizer. Similarly Cd treatment increased the nuclear size after isolating nuclei from CHO cells (Rehak et al. unpublished results). Following a lag period effecting both replicative and repair synthesis in early S phase, DNA replication was supressed throughout the S phase as expected upon DNA damage. To the contrary repair synthesis increased and in early-mid S phase was about 30-fold higher after Cd treatment than in the control population belonging to the same subphase of cell cycle. There is an other 4-fold repair maximum in late S phase. These repair peaks confirm the notion that repair activity is not evenly distributed in the cell-cycle and S phase consists of more subphases what we S phase checkpoints. What we do not know at the moment is whether different types of DNA damages affect different S subphase checkpoints.

The comparison of repair activity with the distribution of strand breaks indicates that peaks of repair activity and those of strand breaks do not coincide. This could mean that strand breaks are necessary but not satisfactory requirements to initiate repair activity. The fluctuation of

naturally occuring strand breaks in control cells during the cell cycle can be explained by the a change of replication intermediates belonging to disconinuous DNA synthesis, such as Okazaki fragments, short replicative intermediates [24] and large DNA fragments accumulating in G2 phase. The number of strand breaks in damaged cells was 10-40 times higher than in control cells showing a cell cycle dependent fluctuation. The number of strand breaks was particularly high at the beginning and at the end of S phase. However, at the beginning of S phase repair activity was low. This discrepancy could indicate that not all of the breaks are being repaired. To replace the catalitically active DNA polymerase to the one which is specific for the damage may postpone repair activity to the next subphase. It may well be, that damaged cells undergo a selection process provided by different types of repair activities and those cells which escape these repair processes or cannot be repaired undergo apoptosis. Our experiments in murine preB-cells after γ-irradiation indicate that the genotoxic stress augmented base excision repair in early S phase and predominantly induced apoptosis at the G2/M checkpoint (Offer, H., Zurer, I., Banfalvi, G., Rehak,M., Goldfinger, N & Rotter, V. unpublished).

Based on overshooting repair and supressed replication peaks an increase in the level of the carcinogenic indicator upon Cd treatment was expected. Indeed 30-60 times elevated levels of 8OHdG were measured after Cd treatment in elutriated fractions [29]. This level was highest in G1 and early S phase and gradually declined by the end of S phase. The decrease of carcinogenic indicator can be explained by 1. the gradual removal of modifyed bases by the end of S phase or. 2. cells in early S phase are more suscepible to Cd treatment in early S phase. Increased base excision repair in early S phase favours the first explanation (Offer et al. unpublished).

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