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FRAGILITY OF TELOMERES AFTER BLEOMYCIN AND CISPLATIN COMBINED TREATMENT MEASURED IN HUMAN LEUKOCYTES WITH THE COMET-FISH TECHNIQUE

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The aim **of the present study was the comparative investigation of action of widely applied anticancer preparations: cisplatin (*cis*-DDP) and bleomycin (BLM) on total DNA and telomeres damage in human blood cells.** ***Methods:*** **The "Comet-FISH technique" – single cell gel electrophoresis ("comet assay") in combination with fluorescent *in situ* hybridization (FISH) was used for this purpose. This newly applied combined approach permits to detect on the same specimen the total DNA damage in individual cells and evaluate specific DNA sequences as well. Telomere – specific – PNA (peptide nucleic acid) probes were used for the localization of telomeres in the comet's head and their migration to the tail.** ***The results*** **obtained indicate that in control variants, due to DNA metabolism and handling, approximately 7% of the DNA and 17% of the telomeres were found in the tail. In cells treated with BLM alone, telomeres leak out with equal probability as total DNA. In turn, the combination of *cis*-DDP with BLM reduces telomere migration more than the migration of total DNA due to *cis*-DDP crosslinking effect. Thus, preferentially telomeric action of the *cis*-DDP can be concluded.** ***Conclusion:*** **The Comet-FISH approach permitted us to reveal the induction of DNA breaks with BLM and its modification due to platinum-crosslink formation, using telomeric PNA probes.**

Key Words**: Comet-FISH, telomere, bleomycin, cisplatin, human blood leukocytes.**

Bleomycin (BLM) is a radiomimetic antitumoral agent inducing both single- (SSB) and double-strand (DSB) breaks of DNA through abstraction of the deoxyribose C4'-H [1, 2]. According to the data of Benitez-L. Bribiesca and P. Sanchez-Suarez [3], BLM treatment produced a moderate increase of SSB preliminary at lower concentration and a striking increase of DSB at higher concentration in normal human lymphocytes that coincided with the presence of apoptosis and DNA ladders. The DNA and telomeres damage induction by BLM in human blood cells by Comet-FISH assay was investigated previously in our work [4].

The attempts to lower the toxicity of BLM were performed. Amifostine (WR-2721) appeared to selectively protect healthy leukocytes from action of BLM [2].

Anticancer drug cisplatin, *cis*-diaminodichloroplatinum (*cis*-DDP) was used as radiation dose-modifying agent. It was revealed that *cis*-DDP can inhibit recovery from radiation damage at doses completely non-toxic to normal tissues and permits to achieve high radiosensitization of cells in antitumor treatment [5]. The modifying effect of *cis*-DDP can be due to its inducing predominately DNA-DNA intrastrand crosslinks [6].

Combined treatment of BLM and *cis*-DDP is applied in clinical practice.

The results of our previous study indicated that the Comet-assay with the application of UV-C was a sensitive approach for the detection of DNA crosslinks generated by *cis*-DDP, which efficiently decreased the levels of UV-C induced DNA fragmentation [7]. Different concentrations of *cis*-DDP were applied to estimate the rate of crosslink formation.

In the present investigation the comparative estimation of separate and combined treatment by BLM and *cis*-DDP was realized on the base of earlier obtained results with BLM [4]. We studied not only the total DNA damage but also the telomere involvement, and relation of the level of telomere specific damage to the total DNA damage. The results of D. Suh et al. [8] imply that the stability of human telomere sequence is important in conjunction with the cancer treatment and aging process. The loss of telomeres may lead to the destabilization of the genome. Thus, the investigation of telomeres damage induction by anticancer drugs can be impotant for elaboration of different treatment approaches.

In our study the total DNA damage formation was estimated by the Comet assay (single cell gel electro-

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Abbreviations used: *cis*-DDP — *cis*-diaminodichloroplatinum; BLM — bleomycin; PNA — peptide nucleic acid; FISH — fluorescence *in situ* hybridization; SSB — single strand breaks; DSB — double strand breaks.

phoresis) and the telomere damage was quantitatively detected by combination of the Comet-assay and the fluorescence *in situ* hybridization (FISH), termed Comet-FISH [9–11]. Telomers located outside the former cell nucleus are regarded as damaged, since the size of the chromatin is reduced to such an extent that DNA migration is enabled. The quantification of the telomeres was achieved by counting telomere signals located in the head and tail area.

MATERIALS AND METHODS

Human blood was obtained from 2 healthy female non-smoker volunteers 22 and 25 years old. Blood was treated for 1 h by BLM (Hexal AG, Germany) at 37 °C, in at least three final concentrations. BLM concentrations were chosen according to literature data in order to result in a dose response curve with minimal cytotoxic effects [2].

Comet-assay. The Comet assay was performed in its alkaline version following the protocol of P. Singh et al. [12] and R. Tice et al. [13]. In short, 90 µl of cell/agarose suspension (containing 8 µl of whole blood with 0.9% low melting point agarose, Type VII, Sigma-Aldrich) were distributed onto frosted microscope slides (Labcraft, London, UK) precoated with ground layer agarose (Type II, Sigma-Aldrich): 1.0% normal melting point agarose in PBS (100 mM phosphate) and 400 µl of middle layer agarose: 1.0% normal melting point agarose in PBS (100 mM phosphate). After the agarose solidified, slides were immersed in cold lysis solution (10 mM Tris-(hydroxymethyl)-aminomethane, pH 7.5).100 mM Na_2EDTA, 2.5 M NaCl,1% Triton X-100, pH 10) for at least 60 min at 4 °C. The slides were placed in an electrophoresis chamber containing alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, 4 C, pH 13) for DNA unwinding. After 20 min, the current was switched on, and electrophoresis was carried out at 1.25 V/cm, 300 mA for 25 min. The slides were removed from the electrophoresis chamber and washed once for 10 min with neutralization buffer (0.4 M Tris-HCl, pH 7.5; 0.08 M Tris–Base, pH 7.2). The slides were stained with SYBR-Green (diluted 1 µl/ml and 30 µl/slide). All of the steps of the Comet assay were conducted under dim light. The experiments were reproduced independently at least two times.

Global DNA damage was microscopically quantified using a Zeiss Axioplan microscope, equipped with an HBO 50 and appropriate filter sets for FITC detection (Zeiss, No. 9). Images were recorded using a intensified video camera (Variocam, PCO, Germany) and were captured to a PC running the Komet 4 software package. (Kineticimaging, UK).

Comet-FISH. For Comet-FISH the slides were prepared as described above, but the staining was omitted [14]. Before hybridization the gels were stored for at least 3 days in absolute ethanol at 4 °C for dehydration. The gels were rehydrated in H_2O for 15 min and subsequently the DNA was denatured by incubation in 0.5 M NaOH for 25 min. The denatured DNA was immediately dehydrated in an ethanol series (75, 80 and 95%, 5 min each) and the gels were carefully air dried until all ethanol was evaporated. PNA probes (Telomere PNA FISH Kit/Cy3; DakoCytomation, Denmark) were used accordingly to the instructions of the manufacturer, but since thermal co-denaturation is not possible with the agarose gels the hybridization probe was prewarmed to approximately 80 °C and 10 µl were applied to an area of approximately 20 x 20 mm. The gels were sealed with a plastic coverslip and the slides were placed in a humidified chamber at 37 °C overnight. The next day the slides were placed at room temperature for 30 min before they were placed in 1x rinse solution supplied with the kit, in order to facilitate the removal of the plastic slides. Then the slides were transferred for 2.5 min in a staining char with prewarmed wash solution at 65 °C, without agitation. Then the slides were quenched in cold 1 x PBD. For counter staining the slides were embedded in diluted SYBR Green (Mobitec, Göttingen) including 50% antifade. The number of telomere signals and localization of the signals (comet head or comet tail) were additionally recorded for each cell. Cell numbers scored for Comet FISH ranged from 50 to 100 cells per slide.

RESULTS

***Dose dependent induction of DNA damage by BLM treatment with and without cis-DDP*.** To analysis the effects of the BLM and *cis*-DDP treatment repeated measurements of the total DNA in tail as well as the percentage of telomeres in tail have been quantified. The experimental details as well as the measurements values are listed in a Table. The "dose-effects" response for total DNA and telomeres damage induced by BLM in the human blood cells presented in Table was obtained earlier in our work [4].

For a statistical analysis the experiments were performed in duplicate (BLM+ *cis*-DDP treatment) or triplicate (BLM treatment alone). For the detection of the total DNA damage 40 comets were analysed per slide and the mean of medians was calculated for the percentage of DNA in comet's tail and for the L/H (length to height) ratio. The Comet-FISH specimens were imaged simultaneously and 50 (30) comets with the highest number of hybridisation signals were selected for further analysis from the abovementioned explicative experiments.

Table. Overview of the measurements for BLM induced total and telomere associated DNA damage with and without *cis*-DDP

Treatment	BLM (IU/ml)							
	Control	12.5	25	100	Control	12.5	25	100
Cis-Pt 3 x 10^{-4} M					+	+	+	+
Comet-assay (N cells)	120	120	120	120	80	80	80	80
Mean [% DNA in Tail] ± SE	7.5 ± 0.2	24.4 ± 0.3	37.6 ± 0.3	38.2 ± 0.3	4.8 ± 0.4	12.1 ± 0.2	14.6 ± 0.2	42.4 ± 0.2
Mean [L/H] ± SE	0.6 ± 0.01	1.8 ± 0.01	2.5 ± 0.01	3.0 ± 0.01	0.6 ± 0.01	0.7 ± 0.01	1.1 ± 0.01	2.8 ± 0.01
Comet-FISH (N cells)	50	50	50	50	30	30	30	30
Mean [% hybridisations per comet] ± SE	16 ± 0.7	21 ± 1.4	22 ± 1.5	21 ± 0.7	10 ± 0.5	18 ± 1.3	14 ± 0.9	23 ± 2.2
Mean [% hybridisations in tail] ± SE	17.8 ± 0.8	41.1 ± 0.5	49.5 ± 1.1	52.1± 2.7	13.5 ± 2.3	19.2 ± 1.3	28.1 ± 1.3	43.8 ± 1.6

As it was earlier reported [4] in cells exposed only to BLM a dose dependent increase of the total DNA fragmentation and the number of telomeres in the tail was observed up to a BLM concentration of 25 IU/ml (Fig. 1, *a*). As can be seen from the presented data, the highest concentration of BLM does not lead to the significant further increase of the DNA fragmentation and telomeres found in the tail. The total dose relationship for both parameters investigated can be fitted by an exponential rise function.

The percentage of telomeres in tail exceeds the percentage of total DNA found in the comet's tail in all examined concentrations.

The use of *cis*-DDP (3 x 10^{-4} M) in combination with BLM can significantly reduce the migration of DNA at the lower BLM doses (compare Fig. 1, *b*, to Fig. 1, *a*). The *cis*-DDP in combination with the highest BLM dose (100 IU/ml) does not show any significant effect in reducing DNA detected damage, suggesting the previously mentioned underlying cytotoxic processes ((38.2 ± 0.3)% *vs.* (42.4 ± 0.2)%).

In combination with *cis*-DDP the frequency of telomeres found in the tail was nearly bisected for BLM concentrations up to 25 IU/ml. This effect was also reduced at higher BLM concentrations to no significant differences in the fragmentation rates of the telomeres ((52.1 ± 2.7)% *vs.* (43.8 ± 1.6)%).

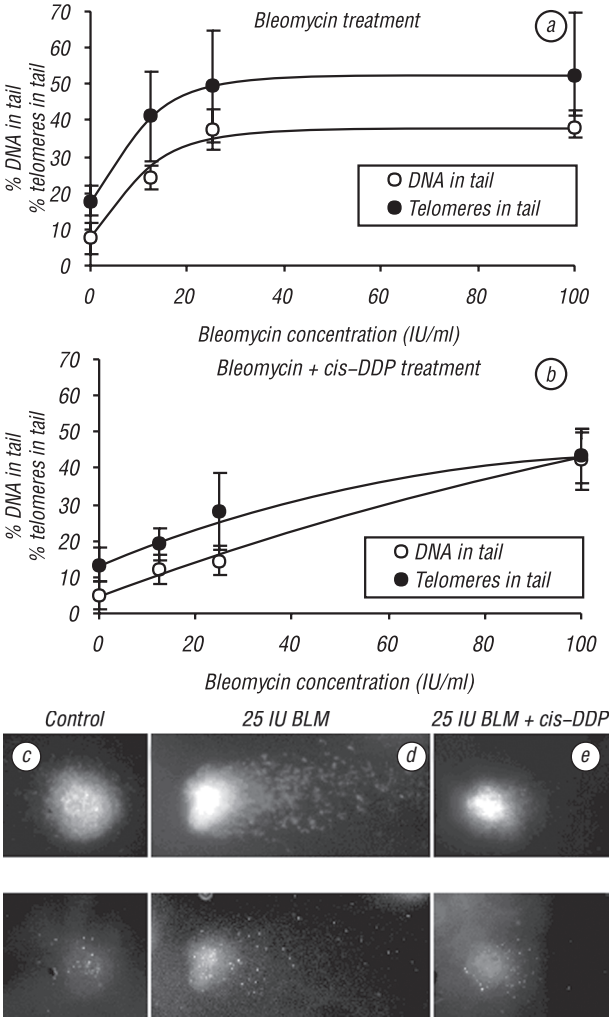

Fig. 1. (*a*) The total DNA damage and the location of telomeres in the tail after BLM treatment. (*b)* Effects of combined *cis*-DDP and BLM treatment on the total DNA damage and the location of telomeres in the tail. (*c–e*) Sample images of Comet-FISH specimens after hybridization of telomere PNA probes. (*c)* Control sells. (*d)* 25 IU BLM treated cells and (*e)* 25 IU BLM + *cis*-DDP treated cells

Fig. 1, *c–e*, shows sample images of Comet-FISH specimens of control cells with nearly all hybridisation signals in the head, a moderate damages cell with multiple signals in the tail as well as a comet after the combined treatment showing reduced DNA migration due to the crosslinking effect of the *cis*-DDP with only a few telomeres in the tail.

To quantify the total DNA damage not only the percentage of DNA in the tail was measured, but also the length to height ratio was computed. This value has a direct correlation to the classification used in [7].

***Total DNA damage induced by BLM compared to telomere associated damage with or without the action of cis-DDP*.** The comparison of the percentage of the total DNA in tail with the percentage of telomeres in tail is shown in Fig. 2. The x-axis gives the detected total DNA damages expressed as mean percentage of DNA in tail. The plotted values on the y-axis represent the mean percentages of telomeres found in tail for each individual concentration. Black circles represent the BLM treatment alone, where in contrast the white circles represent the combinated treatment of BLM with *cis*-DDP.

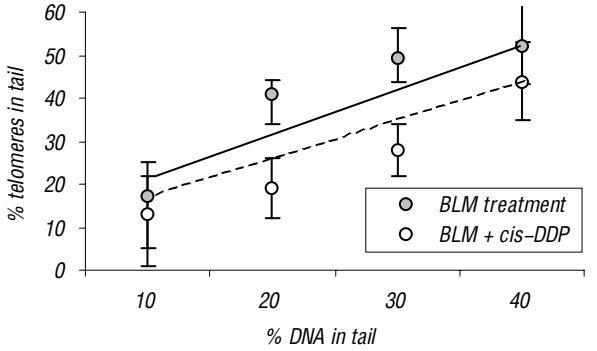

Fig. 2. Direct comparison of total DNA damage versus telomere specific damage. The filled circles represent the mean values of BLM treated samples, whereas the open circles show the mean values for the BLM + *cis*-DDP treated samples

BLM alone reveals a nearly linear correlation between the total DNA damage and the telomere associated damages (fitted slope with b = 1.07). The frequency of telomeres in tail nearly always exceeds the frequency of total DNA in tail.

The use of *cis*-DDP in combination with the BLM reduces the total DNA migration in the Comet-assay. Similarly the frequency of telomeres found in the tail is reduced (compare Fig. 1, *a*, and *b*). The strongest effect is found for 12.5 IU/ml BLM, where total DNA damage is reduced from (24.4 ± 0.3)% to (12.1 ± 0.2)% in the tail, and the percentage of telomeres in the tail is reduced from (41.1 ± 0.5) to (19.2 ± 1.3)% in the tail after the application of *cis*-DDP (see Table).

A direct comparison of the mean values of the overall DNA damage (expressed as the DNA in tail) with the percentage of telomers in tail reveals that in control cells a 17% of the telomeres are located outside of the head (see Fig. 2). In contrast, only 7% of the total DNA is found in

the comet's tail. This hints to a general increased frangibility of the telomeric regions. By comparing the slopes of the linear regressions of the BLM and the BLM-*cis*-DDP treatment a slope of 1.07 was found for the BLM treatment alone, which indicates that the telomeres are of the same sensitivity as the average DNA. In contrast the treatment with the combination of BLM and *cis*-DDP results in a slope smaller than 1 (b = 0.77) and hints to an enhanced cross-linking effect on the telomere sequences.

DISCUSSION

In the present investigation the Comet-FISH approach permitted us to reveal the induction of DNA breaks with BLM and its modification due to platinum-crosslink formation, using telomeric PNA probes. PNA probes were used to detect telomeric DNA repeat sequences in the Comet-assay. By comparing the correlation of total DNA and number of telomeric signals in the comet tail, as a most interesting fact, it could be stated that DNA fragments close to telomeres are found in about the double percentage than are fragments of the total DNA. This is in agreement with the assumption that subtelomeric regions are highly accessible to chromosomal changes.

Therefore a first interpretation of the data leads to the conclusion that telomeric repeats are more fragile compared to the total DNA. But for the interpretation of the data a second fact has to be taken into account: the values for the percentage of telomeres in tail are a pure quantification of the signals found outside of the comets head, as defined by the intensity profile. With a closer look at the specimens two subpopulations of telomeres located in the comet's tail become visible: first the majority of the telomeres can be found only little located outside of the head-area where in contrast a second fraction of signals can be found distributed all over the comet's tail and also at the most distant end of the tail (compare Fig. 1, *c* and *d*).

The first group of signals can be found especially in cells exposed to BLM at low concentrations, where in contrast the second group is increasing with increasing dose. Taking into account that the Comet-assay has a limited resolution of approximately 10–100 kbp using the standard conditions [14] for real DNA fragments and that a part of the DNA located in the tail is composed of so called loops [15, 16], this result can be interpreted in a different way. The telomere signals located closely to the comet's head are probably not caused by localized DNA fragmentation, but represent undamaged telomeres and subtelomeric repeats, which are located outside of the head due to electrostretching effects. Since it is known that telomeres are closely attached to the nuclear membrane [9, 17], the signals located shortly outside of the head can be attributed to this location. In contrast signals found in the distance from the comets head can be assigned to a real fragmentation of the telomeric or subtelomeric repeats. The resolution limit of the Comet-assay, however, may not be neglected in this context: as fragments smaller than 10 kb may be missed by this technique [14], thus breaks within the telomeric repeats are not reliably detected. Taken together the detected fragility of the telomeres, which appears to be higher compared to the total DNA, has to be corrected, since only the fraction of telomeres which are distant from the head to tail border can be counted as broken.

Additionally one has to keep in mind that the telomeres need only one break (within approx. 100 kbp) to generate a fragment, which is able to migrate in the electric field, where in contrast an intrachromosomal fragment needs two hits to form a fragment that is able to migrate outside of the nucleus.

It has to be mentioned, that in the cells treated with *cis*-DDP alone the percentage of telomeres in tail reduced from 17.8 in untreated variants to 13.5 probably due to crosslinks formation.

The combined treatment with BLM and *cis*-DDP changed the results. The percentage of hybridizations in comet tails decreased dose-dependently as did the length of the comet tails, especially at low doses of BLM.

Because *cis*-DDP predominantly induces DNA-DNA intrastrand cross-links, its effect probably can be mostly due to an increase of the size of fragments when they are present in sites of potential breaks [18]. For the cross-linking effect the above mentioned structure of the telomeres is not important. Therefore a preferentially telomeric action of the *cis*-DDP can be concluded. Also, this finding clearly documents that the migration of BLM-induced telomere-bearing DNA in the comet tail is due to actual fragmentation of the DNA strand.

According to our results — the higher the cross-linking effect is the shorter the tails are and the lower is the quantity of telomeres in the comet tails. The reason of this is that the cross-link effect that is more effective at lower BLM concentrations. The presented Comet-FISH approach with telomere PNA permits direct and precise detection of the telomere migration from the former cell nucleus to the comet tail in cells treated with cytostatics, with a direct correlation to the overall DNA fragmentation. That can be important for monitoring the application of these clinical relevant cytostatics during therapy, especially in combinatory approaches, where more than one substance is used at a time. Additionally the results give a detailed insight in the breakage sensitivity of telomeres and subtelomere repeats, which are of great importance for aging and for malignant transformation.

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ИЗУЧЕНИЕ РЕЗИСТЕНТНОСТИ ТЕЛОМЕР МЕТОДОМ COMET-FISH В ЛЕЙКОЦИТАХ ЧЕЛОВЕКА ПРИ КОМБИНИРОВАННОМ ДЕЙСТВИИ ЦИСПЛАТИНА И БЛЕОМИЦИНА

Целью **настоящего исследования было сравнительное изучение действия широко применяемых противоопухолевых препаратов цисплатина (цис-ДДП) и блеомицина (БЛМ) на всю ДНК в целом и на ее теломерные участки в лейкоцитах человека. Была применена техника Comet-FISH — гель-электрофорез единичных клеток (метод ДНК-комет) в сочетании с флюоресцентной гибридизацией *in situ*. Новый комбинированный подход позволяет оценивать на одном и том же препарате как повреждения отдельных клеток ДНК, так и ее специфических участков. Специфичные для теломер PNA-пробы (протеин-нуклеиновая кислота) были использованы для локализации теломер в «голове», а также их миграции в «хвост» кометы. Полученные *результаты* свидетельствуют о том, что в контрольных вариантах, за счет метаболических процессов ДНК и технических условий эксперимента, примерно 7% ДНК и 17% теломер обнаруживаются в хвосте. В клетках, обработанных только БЛМ, теломеры мигрируют в хвост с той же вероятностью, что и вся ДНК. При совместном действие *цис*-ДДП с БЛМ за счет образования сшивок миграция теломер, индуцированная БЛМ, задерживается больше, чем миграция общей ДНК. Таким образом, можно заключить, что *цис*-ДДП более активно действует на теломерные участки. Техника Comet-FISH с применением теломерных PNA-проб позволила оценить индукцию БЛМ разрывов ДНК и их модификацию за счет формирования сшивок цис-ДДП.**

Ключевые слова: **Comet-FISH, теломеры, блеомицин, цисплатин, лейкоциты крови человека.**