CLINICAL-DIAGNOSTIC STUDIES

INTERNALIZATION OF EXTRACELLULAR VESICLES OF CANCER PATIENTS BY PERIPHERAL BLOOD MONONUCLEAR CELLS DURING POLYCHEMOTHERAPY: CONNECTION WITH NEUROTOXICITY

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Extracellular vesicles (EVs), exhibiting their functional activity after internalization by recipient cells, are involved in the pathogenesis of drug-induced polyneuropathy (DIPN), a common complication of antitumor therapy. In this work, the internalization of EVs obtained from colorectal cancer patients undergoing polychemotherapy and its relationship with neurotoxicity were assessed using a model system of mononuclear leukocytes. Circulating EVs were isolated from 8 colorectal cancer patients who received antitumor therapy according to the FOLFOX or XELOX regimens before the start of chemotherapy (point 1) and after 3–4 courses (point 2). Mononuclear leukocytes of a healthy donor served as a cellular model system for EV internalization *in vitro*. EV internalization was assessed using fluorescence microscopy. It was shown that internalization of EVs obtained from colorectal cancer patients with high neurotoxicity was higher than in the group with low neurotoxicity. The ability of CD11b-positive ($CD11b^+$) and CD11b-negative ($CD11b^-$) mononuclear leukocytes of a healthy donor to internalize EVs obtained from patients before and after chemotherapy did not reveal significant differences. A direct relationship was found between the relative number of $CD11b^-$ cells with internalized EVs and the integral index of neurotoxicity according to the NRS scale at the peak of its manifestation (point 2) (r=0.675, p<0.05). By the end of 3–4 courses of polychemotherapy using FOLFOX or XELOX regimens, a tendency towards an increase in the relative number of peripheral blood mononuclear leukocytes (the sum of $CD11b^+$ and $CD11b^-$ cells) with internalized EVs with increased manifestations of DIPN in patients with colorectal cancer was observed.

Key words: extracellular vesicles; internalization; peripheral blood mononuclear cells; colorectal cancer; polychemotherapy; drug-induced polyneuropathy

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INTRODUCTION

Extracellular vesicles (EVs) are nanosized (40–1000 nm) membrane rounded structures of predominantly erythrocyte, platelet, leukocyte and endothelial cell origin; they have been detected in humans in almost all biological fluids (blood, saliva, urine, cerebrospinal fluid) [1]. Currently, much attention is paid to the study of the functional activity of the vesicles, which is believed to be mediated by their internalization by recipient cells. Peripheral blood mononuclear cells (PBMCs) have long been successfully used as a model system to study the internalization of circulating EVs from patients of different age groups [2], patients in a euglycemic state and patients with type 2 diabetes mellitus (T2DM) [3], internalization of EVs from human pancreatic Langerhans cells [4], circulating EVs from patients with glioblastoma multiforme, as well as EVs produced by human anaplastic oligodendroglial cells [5], etc. It has also been shown previously that cells of the monocytic-macrophage series, and to a lesser extent B-lymphocytes, which are the main components of PBMCs, are the main blood cells that internalize EVs of any origin [3, 6]. The ease of PBMC isolation, the possibility of both short-term (up to 2 h) and long-term (up to 48 h) incubation of PBMCs with EVs with maintained their functional activity, the availability of inexpensive modern dyes for EV membrane staining, the possibility of using various inhibitors in the *in vitro* to identify the dominant mechanisms of internalization make the use of PBMCs very attractive to researchers [7]. However, this methodology has not previously been used to search for new available predictors of drug-induced polyneuropathy (DIPN).

It is known that polychemotherapy does not have specificity of action only on tumor cells; so patients may have serious complications from various organ systems due to the toxic effects of antitumor drugs. DIPN as a medical and social problem is quite serious, it develops in more than 70% of patients with the use of a wide range of modern cytostatics and targeted drugs (platinum drugs, taxanes, vinca alkaloids, bortezomib). The severity of DIPN manifestations does not always correlate with the drug dose, and is deteriorated due to the presence of previous polyneuropathy of any genesis. In a significant proportion of cancer patients, especially those receiving taxanes, the severity of DIPN symptoms may deteriorate over time, significantly reducing the quality of life and even reducing the survival rates of the patients [8, 9].

It is also important to note that EVs incubated with recipient cells, can either be internalized into the endosomal compartment with accumulation of EV contents in the cell cytoplasm, or further sorted into lysosomes with subsequent degradation of the contents. In addition, some vesicles can be localized outside the cell in the plasma membrane [7, 10]. A variant of EV internalization into the nuclear compartment has also been described; it may be associated with a change in the functional activity of recipient cells [11].

Many studies have shown that the mechanism and variant of EV internalization depend on both the type and composition of EVs and the recipient cells (cell type, lipid composition, and variant of protein and lipid clustering in the membrane), and age-associated features of EV internalization have been identified; thus, there is a clear need in a certain standardization and unification of these studies [2]. Taking into account the absence of clear predictors of severe DIPN, the clinical importance of the problem of selection, compatibility, duration of drug correction of DIPN, the study of EV internalization in model cell systems is of particular scientific interest.

In this regard, the aim of our study was to assess the internalization of EVs obtained from colorectal cancer patients receiving polychemotherapy and its relationship with the manifestation of neurotoxicity in a model system of mononuclear leukocytes.

MATERIALS AND METHODS

Eight patients (4 women and 4 men, average age 55.5±12.5 years) with colorectal cancer (T3-4N0-2M0-1), who received antitumor therapy according to the FOLFOX or XELOX regimens were included in the study. The chemotherapy FOLFOX regimen included oxaliplatin 85 mg/m^2 as a 2-h infusion on day 1, calcium folinate 400 mg/m^2 (intravenously, i.v.) for 2 h, followed by a bolus administration of fluorouracil 400 mg/m^2 (i.v.) and a 46-h infusion of fluorouracil 2400 mg/m^2. The next course was started on day 15. The XELOX regimen included oxaliplatin 130 mg/m^2 on day 1, capecitabine 2000 mg/m^2 per day on days 1–14. The next course was started on day 22. The average duration of chemotherapy was 2.04±0.35 months.

Table 1 shows patient characteristics, polychemotherapy regimens, and the number of chemotherapy courses received.

Blood for isolation of circulating EVs was collected before the start of chemotherapy (point 1) and at the peak of drug toxicity (point 2), usually after the end of 3–4 courses. At both points, the severity of peripheral polyneuropathy clinical manifestations was assessed using the Numeric Pain Rating Scale (NRS) (patients were asked to rate pain severity using a scale from 0 to 10). The following symptoms were assessed: tingling pain, burning sensation, painful cold sensation, electric shock sensation, crawling sensation, numbness. The tables and figures present the integral pain index. In accordance with the recommendations for the use of the "Common Terminology Criteria for Adverse Events (version 5.0, 2017)" of the US National Cancer Institute [11], groups of patients with no DIPN/mild polyneuropathy (the integral NRS score <4) and with severe DIPN (integral NRS score ≥4) were formed [12].

Small EVs were isolated from the blood plasma of patients by ultrafiltration with double ultracentrifugation (OptimaXP ultracentrifuge, Beckman Coulter, USA). Fractionation of blood cells (about 18 ml of venous blood stabilized with EDTA) was performed using a high-speed centrifuge with an angle rotor at 4°C for 20 min at 1000 *g* (TGL-24MC, Drawell, China). The plasma obtained after the first centrifugation was centrifuged again at 4°C, 20 min, 13000 *g*. The supernatant was diluted with PBS (prepared using buffer solution tablets, Helikon, Russia) and filtered through PES filters with a pore diameter of 220 nm (Nest, China). The filtrate was ultracentrifuged twice in an ultracentrifuge with a swing-bucket rotor (Optima XPN 80, Beckman Coulter) at 100000 *g* for 90 min at 4°C. The resulting aliquots of small EVs were stored at -80°C.

The morphology of the isolated particles was confirmed using transmission electron microscopy. Vesicles isolated from blood samples were adsorbed

Table 1. Characteristics of patients, polychemotherapy regimens, and number of CT courses

No.	Patient's gender	Age, years	T2DM	Cancer stage (TNM)	Regimen of CT	Number of CT courses
1	Female	43	–	T4N2M1	FOLFOX	4
2	Male	47	–	T3N1aM0	XELOX	3
3	Female	72	+	T3N1cM0	XELOX	3
4	Female	68	–	T3N2M0	FOLFOX	4
5	Female	72	+	T4NxM0	FOLFOX	4
6	Male	43	–	T4aN2M1	FOLFOX	4
7	Male	39	–	T4aN0M0	FOLFOX	4
8	Male	60	+	T4aN1aM1	FOLFOX	4

T2DM – type 2 diabetes mellitus; CT – chemotherapy.

for 1 min on copper grids coated with a carbonized film. The grids were then exposed to 0.5% uranyl acetate for 5–10 s. The preparations were examined using a Jem1400 microscope (Jeol, Japan). The hydrodynamic size and concentration of the isolated vesicles were studied using nanoparticle tracking analysis (NTA) by means of a NanoSight LM10 device (Malvern Instruments, UK). Vesicle samples were preliminarily diluted with PBS in proportions of 1:100, 1:1000, and 1:10,000. The average hydrodynamic diameter (vesicle size, their distribution) and vesicle concentration were analyzed. To confirm the vesicular nature of the isolated particles, the level of tetraspanins CD81, CD63, and glycoprotein CD24 was studied by flow cytometry with vesicle enlargement on latex particles. Cytometry was performed on a Cytoflex device (Beckman Coulter); the obtained data were analyzed using CytExpert 2.0 Software. The median fluorescence intensity (MFI) of EVs was analyzed in comparison with isotype and negative controls.

Mononuclear leukocytes were isolated from the peripheral blood of a conditionally healthy donor without oncopathology (52 year old) by sedimentation in a single-step Ficoll density gradient. About 50 μl of EVs were stained with a vital dye for cell membranes PKH26 in accordance with the manufacturer's instructions (LumiTrace PKH Cell Membrane Labeling Kits manual, Lumiprobe Corp., China). A mixture of mononuclear cells with EVs was prepared to set up the internalization reaction. Vesicles labeled with PKH26 and washed from blocking serum (about 600–700 μl) were incubated for 2 h at 37°C (Bios BW-5 water bath; Russia) with 100 μl of PBMCs suspension (1 million cells per ml). To block nonspecific binding, the mixture was incubated with FC block antibody (Elabscience, China) for 15 min, and then the cells were washed in PBS for 10 min at 1000 *g*. The cells were incubated with primary antibodies to CD11b (E-AB-F10081A, Elabscience) for 20 min and after washing in PBS, the nuclei were stained with Nuclear Blue reagent (ThermoFS, USA), 1 drop per sample. Secondary antibodies anti-rat c Alexa 488 (DF7421-F488, Affinity Biosciences, China) were added at the recommended dilution of 1:400, the cells were washed once with PBS; the whole sediment was transferred to glass with an adhesive coating (Superfrost Plus, ThermoFS), the smear was dried, fixed in isoprenol for 1 min. Simultaneously, control samples (a mixture of mononuclear leukocytes was stained without the addition of EVs according to the method presented above) and a control smear containing only stained vesicles were prepared. Evaluation of EV internalization was carried out by fluorescence microscopy (fluorescence microscope Axio Imager. M2, Carl Zeiss, Germany). The total number of mononuclear leukocytes in the blood, as well as $CD11b^-$ and $CD11b^+$ cells with and without signs of EV internalization was counted per 100 cells.

Statistical processing was performed using the Microsoft Office Excel-2021, GraphPad Prism 8, and Statistica 10 for Windows programs. Differences were considered statistically significant at $p<0.05$. The normality of the distribution of the studied samples was checked using the Shapiro-Wilk test. To assess the statistical significance of feature variations (cell populations) in the measurement dynamics, the non-parametric Wilcoxon test for paired samples was used. Parameters in the groups with low and high neurotoxicity were compared using the non-parametric Mann-Whitney test. Non-parametric correlation analysis was performed. Table 2 shows the Spearman rank correlation coefficients.

RESULTS

The integral index of neurotoxicity significantly increased in most patients. Neurotoxicity before the start of polychemotherapy was due to the presence of T2DM and manifestations of diabetic polyneuropathy (Fig. 1).

The main characteristics of the isolated circulating EVs are shown in Figure 2.

All preparations contained EVs of different sizes; some vesicles (Fig. 2A) had a morphology which identified them as small EVs (40–200 nm in size, rounded or cup-shaped). NTA analysis has shown the absence of vesicles larger than 200 nm in the samples. In patients with colorectal cancer, the average vesicle size was 94.0±2.50 nm (Fig. 2B). Binding of labeled antibodies to specific tetraspanin markers CD81, CD63, and CD9 to vesicles (Fig. 2C) confirmed that the vesicles belonged to small EVs.

The isolated PBMC preparations contained both CD11b-positive ($CD11b^+$) and CD11b-negative ($CD11b^-$) cells. At both time points of the study, EVs freely lying outside the mononuclear leukocytes and their conglomerates were also visualized (Fig. 3).

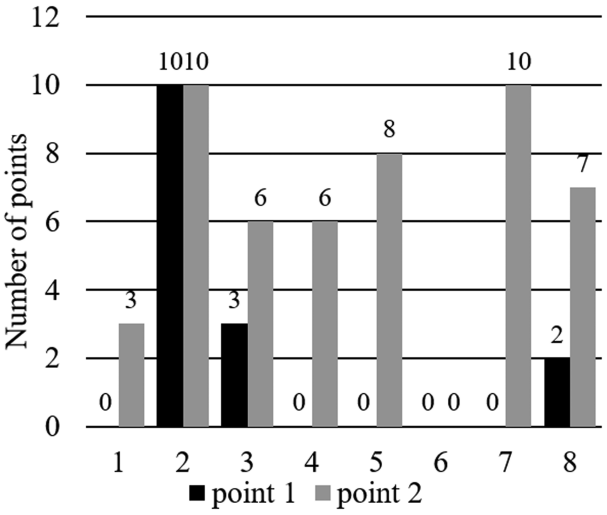

Figure 1. Integral parameter of DIPN severity according to the NRS scale.

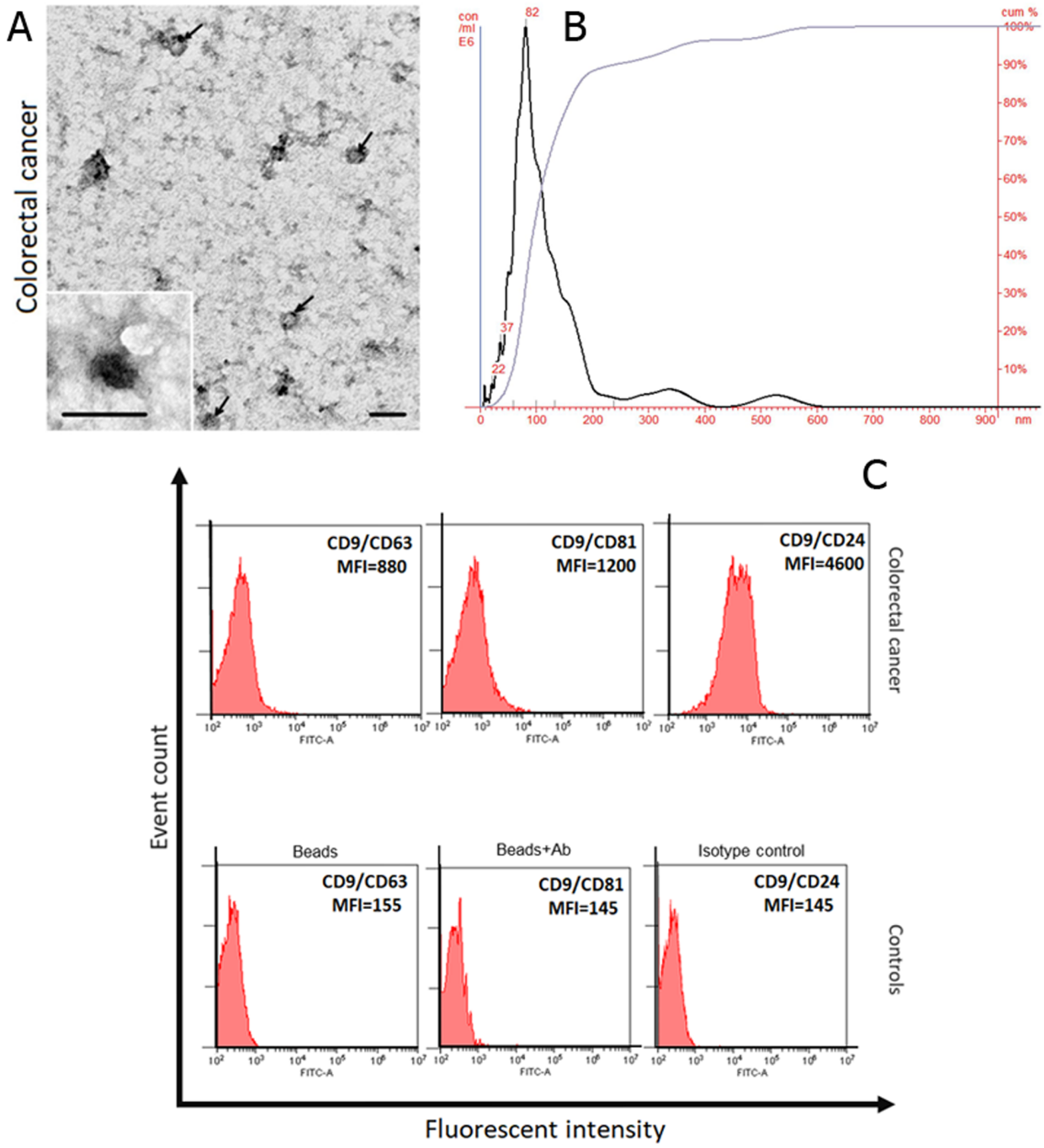

Figure 2. Identification of isolated EVs. **A** – transmission electron microscopy shows the presence of vesicles no larger than 200 nm, the inset shows small EVs, scale lines correspond to 100 nm; **B** – distribution of EV sizes isolated from patients with colorectal cancer and assessed using NTA; **C** – expression of CD63, CD81, and CD24 on $CD9^+$ vesicles, representative values of mean fluorescence intensity (MFI), flow cytometry.

The ability of $CD11b^+$ and $CD11b^-$ mononuclear leukocytes of a healthy donor to internalize EVs obtained from patients before and after chemotherapy did not reveal any significant differences.

Using the *in vitro* model system, we have shown that the internalization of EVs obtained from colorectal cancer patients with high neurotoxicity was higher (at the level of statistical tendency, p=0.0667) than internalization of EVs obtained from colorectal cancer patients with low neurotoxicity (Fig. 4C).

At time point 2, a tendency towards an increase in the relative number of mononuclear leukocytes with internalization of EVs obtained from patients with increased drug-induced neurotoxicity was noted: in patients with no/mild DIPN, the average number of mononuclear leukocytes with internalization (the sum of $CD11b^+$ and $CD11b^-$ cells with internalization) was 12.24% (median 14.61%), and in the group with pronounced DINP it was 23.62% (median 29.12%) (Fig. 4).

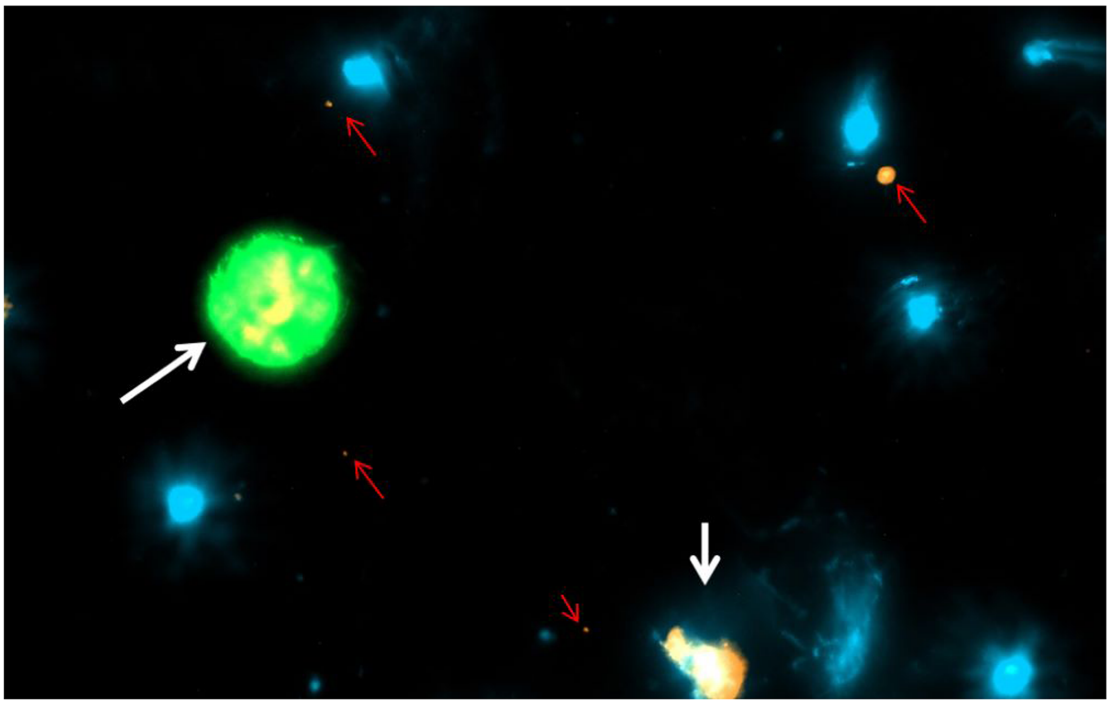

Figure 3. Micrograph of internalization of extracellular vesicles by mononuclear cells *in vitro*, fluorescence microscopy (×400). White arrow shows $CD11b^+$ cells (green label) with internalized EVs and $CD11b^-$ cells with EVs internalized into the perinuclear space. Red arrow shows EVs without internalization.

Table 2. Spearman correlation coefficients (r) at the achieved significance level of $p<0.05$ in patients with severe DIPN at point 2

Parameters	**NRS**	**$CD11b^+$ with int., %**	**$CD11b^-$ with int., %**
NRS	1.000	-0.048	0.675
$CD11b^+$ with int., %	-0.048	1.000	-0.024
$CD11b^-$ with int., %	0.675	-0.024	1.000

NRS is an integral parameter of neurotoxicity according to the NRS scale, $CD11b^+$ with int. – $CD11b^+$ cells with internalized EVs, $CD11b^-$ with int. – $CD11b^-$ cells with internalized EVs.

In order to identify cell subpopulations in which changes in the dynamics of chemotherapy were associated with manifestations of neurotoxicity, a correlation analysis was performed. A direct relationship was found between the relative number of $CD11b^-$ cells with internalized EVs and the integral neurotoxicity index according to the NRS scale at the peak of neurotoxicity (point 2) (r=0.675, p<0.05) (Table 2).

DISCUSSION

Since the functional activity of circulating EVs is suggested to be mediated by their internalization by recipient cells, we have investigated the internalization of EVs isolated from blood of colorectal cancer patients at the stages of polychemotherapy. PBMCs from a healthy donor were used as recipient cells, and the internalization results were analyzed in relation to the manifestation of DIPN. It was previously shown that EVs were effectively internalized by neurocytes, glial cells, including Schwann cells, via endocytosis and macropinocytosis. They contain functionally active proteins (receptors, cytokines, growth factors, enzymes), non-coding RNA and can participate in the pathogenesis of DIPN [13, 14]. However, for example, exosomes from multipotent mesenchymal stem cells carrying a large number of trophic factors have been successfully used to treat diabetic peripheral neuropathy [15, 16]. It is believed that the internalization of EVs by neurons and neuroglia is the most critical moment for the therapeutic effect of EVs. It has been shown that intravenous administration of small EVs from normal Schwann cells significantly ameliorated the course of peripheral neuropathy in diabetic mice [13]. Although a number of molecular markers (polymorphic genes TLR6, IL4, IL-1β, mutations in the genes IL10 and IL2) have been proposed as potential molecular markers of bortezomib-induced polyneuropathy in patients with multiple myeloma [17], vincristine-induced polyneuropathy in children [18] and taxane-induced polyneuropathy [19], the search for these markers in available biological fluids is certainly relevant at present. EVs, widely represented in blood plasma, are currently considered as a source of these markers.

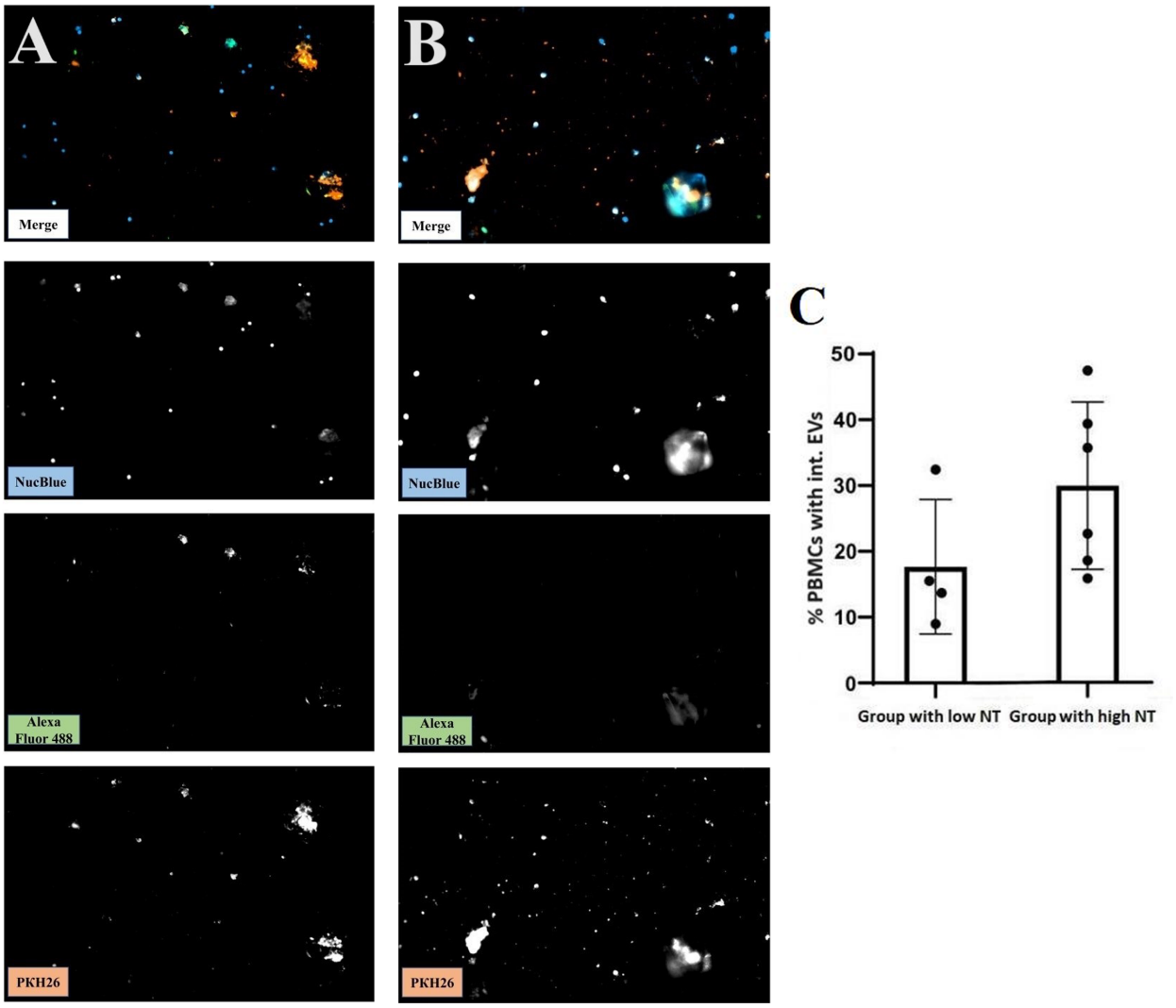

Figure 4. Internalization of EVs obtained from the blood of patients (point 2) with low (**A**) and high (**B**) degrees of neurotoxicity, fluorescence microscopy (×200). Percentage of mononuclear leukocytes with internalization of EVs obtained from patients with low and high neurotoxicity (**C**). Comparison of groups by means of the nonparametric Mann-Whitney test, p=0.0667.

Our study revealed a direct relationship between the number of CD11b- mononuclear leukocytes with internalized EVs before treatment and the NRS scale integral neurotoxicity index at the peak of neurotoxicity (3–4 cycles of polychemotherapy) (r=0.675, p<0.05). In our opinion, the expansion of the sample will allow us to identify and validate objective DIPN predictors in the future. The advantages of the proposed method include the simplicity and standardization of the isolation of PBMCs, the classical cells, which predominantly internalize EVs. In addition it is rather cheap and rapid: the entire procedure from EV staining to visualization of cells with *in vitro* internalized EVs in the preparation takes no more than 6–7 h.

CONCLUSIONS

A tendency towards an increase in the relative number of mononuclear leukocytes in the peripheral blood (the sum of $CD11b^+$ and $CD11b^-$ cells with internalization) with an increase in the manifestations of drug-induced polyneuropathy in patients with colorectal cancer was noted by the end of the 3–4 courses of the FOLFOX or XELOX polychemotherapy regimens. A direct relationship was found between the number of $CD11b^-$ cells with internalized EVs before treatment and the integral neurotoxicity index according to the NRS scale at the peak of neurotoxicity (after 3–4 cycles of chemotherapy). Expanding the sample will allow in the future to identify and validate objective predictors of drug-induced polyneuropathy.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Patients and a healthy volunteer gave written informed consent for the use of their biological material in the study. The study was conducted in accordance with the Helsinki Declaration and approved by the Ethics Committee of the Cancer Research Institute of the Tomsk National Research Medical Center (Protocol no. 10 dated 10.02.2022).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ИНТЕРНАЛИЗАЦИЯ ВНЕКЛЕТОЧНЫХ ВЕЗИКУЛ ОНКОЛОГИЧЕСКИХ БОЛЬНЫХ МОНОНУКЛЕАРАМИ ПЕРИФЕРИЧЕСКОЙ КРОВИ ПРИ ПРОВЕДЕНИИ ПОЛИХИМИОТЕРАПИИ: СВЯЗЬ С НЕЙРОТОКСИЧНОСТЬЮ

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Внеклеточные везикулы (ВВ), функциональная активность которых, по-видимому, опосредуется их интернализацией клетками-реципиентами, вовлечены в патогенез лекарственно-индуцированной полинейропатии (ЛИПНП) — частого осложнения противоопухолевой терапии. В данной работе на модельной системе мононуклеарных лейкоцитов оценена интернализация ВВ, полученных от больных колоректальным раком в условиях проведения полихимиотерапии, и её связь с проявлением нейротоксичности. Циркулирующие ВВ выделены от 8 больных колоректальным раком, получивших противоопухолевую терапию по схемам FOLFOX или XELOX до начала химиотерапии (точка 1) и после 3–4 курсов (точка 2). Мононуклеарные лейкоциты здорового донора служили в качестве клеточной модельной системы для интернализации ВВ *in vitro*. Оценку интернализации ВВ проводили методом флуоресцентной микроскопии. Показано, что интернализациия ВВ, полученных от больных колоректальным раком с проявлением высокой нейротоксичности, была выше, чем в группе с низкой нейротоксичностью. Способность CD11b-позитивных ($CD11b^+$) и CD11b-негативных ($CD11b^-$) мононуклеарных лейкоцитов здорового донора интернализировать ВВ, полученные от больных до проведения химиотерапии и после, не обнаружила значимых различий. Выявлена прямая взаимосвязь между относительным количеством $CD11b^-$ клеток с интернализованными ВВ и интегральным показателем нейротоксичности по шкале NRS на пике её проявления (точка 2) (r=0,675, p<0,05). К окончанию 3–4 курса полихимиотерапии по схемам FOLFOX или XELOX отмечена тенденция увеличения относительного количества мононуклеарных лейкоцитов периферической крови (сумма $CD11b^+$ и $CD11b^-$ клеток) с интернализованными ВВ с усилением проявлений ЛИПНП у больных колоректальным раком.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: внеклеточные везикулы; интернализация; мононуклеарные лейкоциты периферической крови; колоректальный рак; полихимиотерапия; лекарственно-индуцированная полинейропатия

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