MOLECULAR-BIOLOGICAL PROBLEMS OF DRUG DESIGN AND MECHANISM OF DRUG ACTION

ROLE OF PRE- AND POST-REPLICATIVE MISMATCH REPAIR IN CYTOTOXICITY OF METHYLATING ANTITUMOR AGENTS (A REVIEW)

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The review considers modern data on the pre- and post-replicative repair of DNA damage induced by methylating agents such as *N*-methyl-*N*-nitrosourea, temozolomide, procarbazine, dacarbazine, and aranoza. These drugs are used in the treatment of various types of tumors including Hodgkin's disease, brain tumors, disseminated melanoma, and lymphoproliferative diseases. Resistance (both intrinsic and acquired) to methylating agents is an important problem in cancer chemotherapy. The cytotoxicity of methylating agents depends on O^6-methylguanine-DNA-methyltransferase (MGMT) activity (prereplicative repair). Several preclinical and clinical studies have demonstrated that postreplicative mismatch repair (MMR) is responsible to a high degree for the tumor cell resistance to the methylating agents. MMR in experimental studies is determined using expression of the main proteins hMLH1, hMSH2, and hMSH6 involved in the activity of the MMR system. Resistance to methylating agents is due to hypermethylation of promoters of the corresponding genes. The deficiency of hMLH1, hMSH2, and hMSH6 in tumors and lymphocytes after pre-operative neoadjuvant chemotherapy may serve as an independent predictor of poor prognosis in the development of disease.

Key words: prereplicative repair, MMR, antitumor methylating agents

DNA-methylating agents such as *N*-methyl-*N*-nitrosourea (MNU) [1], 8-carbamoyl-3-methylimidazo[5.1-α]-1,2,3,5-tetrazin-4-(3H)-1 (temozolomide), *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide (dacarbazine), 5-(3,3-dimethyl-1-triazenyl-1H-imidazol-4-carboxamide (procarbazine) [2], and 3-α-L-arabinosyl-1-methyl-1-nitrosourea (aranoza) [3] are used in combination chemotherapy to treat several cancerous diseases (disseminated melanoma, small-cell lung cancer, lymphoproliferative diseases, glioma). The high antitumor activity of these compounds that was found in experimental studies has not been fully realized in clinical settings because of intrinsic and acquired drug resistance during the treatment.

Prereplicative repair of DNA damage and resistance to methylating agents. It has been found that the cytotoxicity of methylating agents depends to a high degree on the extent of methylation of guanine in the O^6 position and the lifetime of this modification, which in turn depends on the activity of the acceptor protein O^6-methylguanine-DNA-methyltransferase (O^6-MGMT). The gene coding O^6-MGMT is known to be inactivated in several human tumors such as B-cell lymphoma and brain tumor owing to epigenetic mutations, mainly, hypermethylation of the promoter region of this gene [4, 5]. Clinical observations showed that these tumors are sensitive to methylating agents [1]. The mechanism of action of O^6-MGMT is unique because this protein in eucaryote cells transfers methyl groups from O^6-MG in DNA onto an active cysteine within its own sequence. This forms *N*-methylcysteine and restores the

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DNA guanine. The properties of O^6-MGMT have been reviewed [6, 7] and discussed in other publications. The high level of O^6-MGMT in tumor cells enhances the development of resistance to methylating agents. Determination of O^6-MGMT activity in 100 strains of human tumors showed that only 20% are sensitive to these compounds and typically have a low level of O^6-MGMT. Low activity of O^6-MGMT and high sensitivity to methylating agents is observed in most tumors in experimental animals [8, 9]. This may be one reason for the different sensitivity of human and animal tumors to methylating agents.

Attempts were made to overcome resistance to methylating agents using inhibitors capable of depleting the pool of O^6-MGMT. This complicates prereplicative repair and creates conditions conducive to cytotoxicity of methylating agents. The most common inhibitor of O^6-MGMT is the pseudosubstrate O^6-benzylguanine, which is currently used in combination with methylating antitumor drugs in the clinic to treat gliomas and certain other cancerous diseases [10].

Mismatch repair (MMR) and resistance to methylating agents. Concepts about the involvement of postreplicative MMR of incorrectly paired DNA bases in apoptosis induced by methylating agents have recently been rapidly developing. It was noted first in 2000 [11] that the functioning of only prereplicative repair of DNA induced by methylating agents could not explain apoptosis of tumor cells. O^6-MG is paired with thymine during replication. This activates the MMR system, a multicomponent system consisting of proteins hMSH2 and hMSH6 that form a heterodimer (hMut-Sα) capable of recognizing incorrectly paired DNA bases (initiation stage). Then hMSH2-hMSH6 forms a complex with another heterodimer, hMLH1 and hPMS2 (hMutLα). This leads to further initiation of MMR. Exonuclease (Exo1), which proliferates a nuclear antigen (PCNA), DNA-polymerase ε and δ, and DNA helicase1, the roles of which have not yet been thoroughly studied, are involved in the last stages (excision and resynthesis).

Expression of main proteins hMLH1, hMSH6, and hMSH2 is currently determined by immunohistochemical studies of the functioning of the MMR system in chemotherapy. It was hypothesized that not only the level of expression of O^6-MGMT but also defects in the MMR system are the main factors in the resistance of tumors to methylating agents [12]. Then this hypothesis was confirmed in *in vitro* and *in vivo* experiments.

MMR-deficient tumor cells are about 100 times more resistant to methylating agents of the S_N1 type than to other agents. Antitumor drugs such as mitomycin, chloroethyl nitrosourea, melphalan, etoposide, and cisplatin have only a slight effect on the MMR system [13, 14]. However, with respect to chloroethyl nitrosourea and cisplatin, their effect on the MMR system is not fully known. Thus, experiments on cultures of human ovarian tumors showed that MMR-deficient strains were three times more resistant to CCNU than MMR-proliferative strains [15]. However, contradictory results were obtained by the same group using another experimental model [16].

Experiments with cultures of melanoma cells showed that the level of O^6-MGMT and the toxicity of temozolomide were directly correlated in MMR-deficient cells. However, the sensitivity of melanoma cells to temozolomide increased and depended only on the effectiveness of the MMR system if O^6-MGMT was fully inhibited by the specific inhibitor O^6-benzylguanine. MMR-deficient cells were practically completely resistant to temozolomide regardless of the activity level of O^6-MGMT [17].

Furthermore, it was found that expression of the hMutLα protein complex is silenced in certain lines of melanoma cells with MMR defects [18]. Addition to extracts of melanoma cells deficient in MMR of purified recombinant protein hMut-sα but not hMutlα restored 90% of the MMR functional activity. The ineffectiveness of the MMR system in these cells was probably partially due to defects in hMSH2 and hMSH6 (heterodimer hMut-Sα). Methylation of DNA results in the formation of O^6-MG-T and O^6-MG-C pairs that are recognized by hMut-Sα protein with subsequent activation of the MMR system [17].

The cytotoxicity of methylating agent temozolomide was determined using athymous mice with grafted MMR-deficient and MMR-producing human glioma cells. The activity of base excision repair (BER) enzyme poly(ADP-riboso)polymerase (PARP) was determined simultaneously for these models [19]. As it turned out, inhibition of this enzyme by isoindoline (INO-1001) enhances the cytotoxicity of temozolomide, especially in MMR-deficient cells [20]. An analogous effect was obtained using other PARP inhibitors such as AG-1436 and ABT-888 [20 – 22]. It was thought that the antitumor activity of this drug is due to not only methylation of guanine in the O^6-position but also the formation of other adducts such as N^7-MG and N3-methyladenine because inhibition of PARP in MMR-producing and espeically MMR-deficient cells enhances the cytotoxicity of temozolomide. Attempts were made to restore the MMR system functioning in MMR-deficient cells. It was assumed that decitabine prevents hypermethylation of the hMLH1 promoter gene [23].

There is at present much experimental evidence linking drug resistance to defects in hMLH1 and hMSH2 that hinder functioning of the MMR system. This makes it possible to create drugs that increase the effectiveness of chemotherapy of cancerous diseases.

Possible use of MMR for predicting the effectiveness of tumor treatment. It should be noted that the role of MMR in the effectiveness of a drug for treating oncological patients is poorly studied. Reduced expression of the main protein of MMR, hMLH1, in particular as the result of hypermethylation of the promoter gene of this protein, can lead to the development of resistance to antitumor drugs. This hypothesis was confirmed in several clinical investiga-

tions. Expression of hMLH1 and p53 was determined in breast cancer biopsies and tumors of 30 patients after neoadjuvant chemotherapy. As it turned out, expression of these proteins before chemotherapy is not a predictive factor of the treatment effectiveness. Substantial reduction of hMLH1 expression after pre-operative chemotherapy can be used as an independent predictor of poor prognosis. Chemotherapy had practically no effect on p53 expression [24]. Analogous results were obtained in esophageal cancer patients after pre-operative treatment (cisplatin, adriamycin, and fluorouracil) followed by an operation. Expression of hMLH1 was observed in specimens of esophageal cancer. It more than doubled (from 14 to 37%) in 40% of patients after neoadjuvant chemotherapy. A low initial level of expression of this protein is an independent predictor of patient survival, for which the five-year survival rate was 19.3% compared with 41% of patients with a high level of hMLH1 expression. Chemotherapy has no effect on p53 expression and is not related to the predicted treatment effectiveness [25]. Mutations in the hMSH6 gene were not observed before treatment with temozolomide in glioblastoma patients. Expression of MMR protein hMSH6 was noted before chemotherapy in 17 out of 17 instances. After treatment, recurrence of tumors with a notably increased expression of hMSH6 that was accompanied by accelerated growth of glioblastoma tumors was noted in 7 of the 17 patients. A deficit of hMSH6 may be indicative of the possible appearance of recurrent tumors after treatment with temozolomide. Decreased expression of hMSH6 was not related to the status of O^6-MGMT [26].

Contradictory results were obtained in the only study found by us where patients with astrocytoma were treated with bifunctional chloroethyl nitrosourea (ACNU). Tumor samples were taken for analysis of hMLH1 gene during the operation before treatment. It was found that 6 of 41 (15% of patients) had hypermethylation of hMLH1 gene promoter that, according to the report, may be indicative of sensitivity to adjuvant chemotherapy and may be a factor in favorable prognosis of treatment effectiveness. It was shown that hMLH1 protein could not be found in tumors of patients in which the hMLH1 promoter gene was hypermethylated. Nevertheless, these patients were more sensitive to ACNU treatment. It is at present difficult to explain the results of this investigation [27].

Resistance to procarbazine was acquired in children with highly differentiated glioma, in contrast with adult patients. The glioma cells turned out to be deficient in MMR with a high level of O^6-MGMT [28].

Analysis of MMR and apoptosis proteins in lymphocytes of patients before and after treatment is useful for predicting individual sensitivity to chemotherapy. Tumors that were highly sensitive to chemotherapy (Hodgkin's disease, Williams tumor, testicular seminoma, etc.) were selected [29]. Expression of hMLH1 and hMSH2 was substantially increased (by 80%) in lymphocytes of patients with total remission of cancerous diseases.

Thus, the apoptosis initiator O^6-MG is formed after reaction with methylating antitumor drugs that are used in combination chemotherapy to treat disseminated melanoma, brain tumor, small-cell lung cancer, and lymphoproliferative diseases.

O^6-MG forms during replication a mismatched pair of bases with thiamine O^6-MG-T that activates the MMR system. Resistance to methylating agents, which limits the effectiveness of chemotherapy, depends on the protein-transporter O^6-MGMT (prereplicative repair). Prereplicative repair is currently well studied. The O^6-MGMT inhibitor benzylguanine is already used in the clinic. The significance of various defects in the functioning of the MMR system and their role in the appearance of resistance to methylating antitumor drugs remain unclear. The timeliness of investigating the disruption of MMR protein expression (hMut-Sα and hMutLα), exonuclease I, replication A protein, proliferating nuclear antigene PCNA, and replication C factor is obvious. New information in this area may help to predict the effectiveness of drug treatment of cancerous diseases.

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