Role of Different Isoforms of Nitric Oxide Synthase in Development of Tumor Mutants in *Drosophila melanogaster*

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Abstract—We studied the role of nitric oxide synthase during tumor growth in oncovirus-induced tumor mutants of *Drosophila melanogaster*. The lines with different capacity for malignancy differed reliably in the level of enzymatic activity. It was shown using specific inhibitors of neuronal and inducible isoforms that the neuronal isoform was not involved in tumor formation, while the inducible one appears to play an important role in tumor growth inhibition. This isoform was identified with the help of immunoblotting and monoclonal antibodies against inducible nitric oxide synthase.

Key words: nitric oxide, nitric oxide synthase, neuronal isoform, inducible isoform, *Drosophila*, malignancy, nitric oxide synthase inhibitors, monoclonal antibodies.

Studies of the role of signal molecules in the regulation of physiological and pathological processes, in which these molecules are involved, are very topical. Nitric oxide (NO) is one of these molecules. This compound plays the key role in the regulation of neurotransmission, blood clotting, control of blood pressure, formation of mechanisms of thinking, memory, and sleep, capacity of the immune system to suppress the growth of tumor cell, and many other processes (Moncada et al., 1991; Murad, 1999).

The existence of nitric oxide synthase (NOS), and enzyme catalyzing the formation of nitric oxide and L-citrulline from L-arginine, was demonstrated in the end of 1980s and the enzyme was purified by 1990 (Moncada et al., 1989; Stuehr et al., 1989; Bredt and Snyder, 1990). At present, three isoforms of the enzyme have been identified, which differ in cell specificity and mechanisms of regulation: neuronal, endothelial, and macrophagophal (Forstermann et al., 1994; Knowles and Moncada, 1994; Gorren and Maier, 1998). The neuronal (nNOS) and endothelial (eNOS) nitric oxide synthases are constitutive Ca$^{2+}$-dependent forms, while the macrophagophal (iNOS) is an inducible Ca$^{2+}$-independent form. The latter is induced in the course of pathological processes, for example during inflammation or tumor growth, and NO produced by this form is involved in the immune protection of cells (Forstermann et al., 1994; Thomsen and Miles, 1998).

In mammals, these enzymes were extensively studied. But in invertebrates, the systematic studies of NOS activities have begun only recently. The enzyme was shown to be active in tissue extracts of several invertebrates (Ribeiro et al., 1993; Muller, 1997). Nitric oxide was involved in the transmission of signals in some invertebrates, including *Drosophila* (Muller, 1994). The NOS activity was recorded in the brain, optic lobes of insects, salivary glands of blood-sucking insects, and imaginal discs of insects (Gibbs and Truman, 1998). Regulski and Tully (1995) cloned the NOS gene from the *Drosophila* genome library homologous to the rat nNOS gene. These results initiated the studies of the role of nitric oxide in ontogenesis of *Drosophila*.

It was shown that NO-system is involved in the control of epithelial secretion of malpighian vessels in *Drosophila* (Davies et al., 1997). Kuzin et al. (1996) demonstrated that the neuronal isoform NOS was expressed at a high level in the developing imaginal discs. Inhibition of nNOS in *Drosophila* larvae induced the hypertrophy of organs, while in the presence of nNOS inducers, the adult fly organs were underdeveloped. Those authors concluded that NO acted as an antiproliferative agent during development of *Drosophila* and controlled the balance between cell proliferation and differentiation (Kuzin et al., 1996).

The results of many studies concerning the role of NOS genes during tumor growth in mammals suggest that NO can act as an important mediator of tumor growth. Directly opposite effects of NO on tumor growth were recorded in different types of tumor cells. Dong et al. (1994) studied the effect of endogenously
synthesized NO on the growth of melanoma cells in vitro and in vivo. If the expression of iNOS was induced by cytokines, mediators of inflammatory process, the cell growth in vitro was reduced the tumor development and metastatic spreading in vivo were restricted. In the cells of human adenocarcinoma (DLD-1) and murine mammary gland carcinoma (EMT-6), which express iNOS like in the melanoma cells, the reduced growth in vitro was also recorded (Xie et al., 1996). However, it has been established that NO stimulates the tumor growth through regulation of the tumor activities in the cells of human erythroleukemia (K562) during in vitro growth (Maccarrone et al., 1998). During chromic inflammation, the combined effect of NO active oxygen forms may enhance carcinogenesis in the man (Bruce et al., 1995). Liu et al. (1994) showed on the mode of chronic hepatitis, viral hepatitis in the marmot, that this infection increases the risk of carcinogenesis in the liver with respect to increased NO generation in this organ. In chronic hepatitis patients, and increased NO level was also recorded, which suggests their predisposition to liver cancer (Taylor et al., 1998. Note, however, that NO synthesized by the activated macrophages is capable of suppressing the growth of cancer cells (Stuehr et al., 1989). Hence, the published data about the NO effects are contradictory.

The aim of this work was to elucidate the role of nitric oxide synthase during tumorigenesis on the model of oncovirus-induced mutant lines of Drosophila melanogaster.

MATERIALS AND METHODS

Drosophila lines used in this study. Studies were carried out on D. melanogaster mutant lines with genetic instability induced by oncoviral DNAs (Nabirochkina et al., 1991):

(1) LTR 10, a lethal of 3rd chromosome, was obtained after injections of LTR (long terminal repeats) RSV under the plasmid pPrC/
 in the Oregon R (Or R) embryos. The larvae homozygous for the lethal die in the end of 3rd larval instar.

(2) Onc 9, a lethal of 3rd chromosome, was obtained from an unstable line after injections of the gene src RSV fragments under the plasmid pPrC/
 in the Or R embryos.

(3) Onc 36, a lethal of 3rd chromosome, was screened from the dp line obtained after injection of the plasmid pPrC11 containing a practically entire RSV genome, except some pol and env genes, in the T-007/Oregon R embryos with the syndrome of hybrid dysgenesis (Gabitova, 1993).

The Oregon R wild type line was used as the control. The flies were cultivated in a thermostat at 25°C on standard nutrient medium No. 1 (Kakpakov, 1977).

Histological methods. In order to study the capacity of mutant larvae tissues for neoplastic growth during in vivo cultivation, eye-antennal and wing imaginal discs were isolated surgically from the 3rd instar mutant lines (72–84 h) in the Beadle-Ephrussi saline and transplanted in the abdomen of adult Oregon R females using a capillary needle. In the control experiments, tissues of Oregon R wild line larvae were transplanted in the abdomen of Oregon R females. The operated flies were examined daily under a dissection microscope to reveal the arising defects in recipient females. Within four to five days, the abdomens of operated flies were isolated surgically and subjected to histological analysis.

The abdomens of flies were fixed by Bouin solution for 1–3 days. After fixation, the objects were washed from the fixative by 70% ethanol for 24 h, dehydrated in ascending alcohols (80 and 96%—30 min, 100%—40 min), kept in a mixture of 100% alcohol and chloroform (1 : 1) for 30 min and in pure chloroform for 40 min. The objects were then transferred in a chloroform-paraffin mixture (14–20 h, 37°C), impregnated in pure paraffin (two changes, 1.5 h each, 56°C), and embedded in paraffin. Sections, 3–5 μm were prepared. The histological preparations were stained by azur II-eosin (Evgen’eva, 1983). The pattern of behavior of the transplanted tissues on these preparations was determined.

Estimation of nitric oxide synthase activity. The synchronized cultures of Drosophila embryos, larvae, pupae, and imago were frozen at –80°C. The samples were homogenized in a 40-fold volume of Na-phosphate buffer (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.0) and incubated at the room temperature for 5 min. The homogenate was centrifuged at 16 000 g for 20 min. Protein was assayed in the supernatant by the standard method (Lowry et al., 1951) and the enzymatic activity was determined using the Griss reagent (10 mM sulfanilamide, 1 mM naphthylethylenediamine, 2 M HCl) on an Ultraspec 2000 spectrophotometer (Pharmacia, Sweden) at the wavelength 550 nm (Misko et al., 1993).

L-arginine-NG-aminoflavinate (Sigma, USA) and S-methyl-L-thiocitrulline (Alexis, USA) were used as specific inhibitors of iNOS and nNOS, respectively. The synchronized cultures were placed in a 1 mM inhibitor solution in Na-phosphate buffer for 10 min, washed by the same buffer, and transferred onto a nutrient medium. The NOS-activity was measured within 5 h.

Immunoblotting (Western-blot analysis). The larval tissues were homogenized in a boiling lysing buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, and 2% sodium dodecylsulfate (SDS)) and the homogenate was boiled for 5 min. Electrophoresis was performed in 8% polyacrylamide gel (PAAG) in the presence of SDS, as described elsewhere (Laemmli, 1970). Protein was assayed after Lowry. In order to determine the molecular mass of proteins, the marker Rainbow™ (Sigma) with the molecular resolution 14.3–200 kDa was used.