Induction of murine malignant lymphoma by 1-(2-chloro-ethyl)-3-cyclo-hexyl-1-nitrosourea, *In vitro* partial characterization

استحداث سرطان اللمفوما الفاريه باستخدام الاثل نايتروزويوريا وانشاء مزرعه خلوية اولية منه

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Abstract:

From 20 mice administered ethyl nitrosourea for over 5 months, 2 mice showed lung lymphoma, one of them is a murine malignant lymphoma which cultured and the cell line was newly established as short term culture. The cells were round in shape and had a tendency to make groups of floating clusters. Later these cells developed to proliferate as adherent cell culture. Our study showed that ethyl nitrosourea is selective carcinogen to induce lymphoma and lymphoma cultured cells is very useful for lymphoma studies.

المستخلص

أظهرت فئرتان من اصل 20 فأر مجرع بمادة الاثيل نايتروزويوريا لمدة خمسة اشهر حالة لمفوما في منطقة الرئة. وكانت عبارة عن حالة لمفوما خبيثة ,حيث زرعت في الزجاج وتم انشاء مزرعه خلويه منها وكان شكل الخلايا كرويا دائريا ولها الميول لتكوين عناقيد طافية من الخلايا . تتكاثر الخلايا وهي طافيه ، تمثل هذه المزرعه الخلويه مادة مهمه لدراسة اللمفوما البشرية . وتظهر الدراسة ان مادة الاثل نايتروزو يوريا هي مسرطن متخصص يمكن استعماله لاحداث اورام اللمفوما في الفئران للدراسات المختبرية .

Introduction:

Lymphomagenesis is a multistage process in which accumulated genetic lesions promote the proliferation and or survival of target cells that eventually generate an autonomous malignant clone [1]. Both oncogene activation and tumor suppressor gene inactivation are implicated in the development of murine lymphomas induced by radiation, chemical carcinogens or oncogenic virus [2]. Lymphoma induced by irradiation as UV irradiation [3], or x-irradiation [4]. Specific gene mutations are associated with lymphoma, [5] found that losing of *Runx1* gene make mice more susceptible to ethyl nitrosourea carcinogenesis which predispose mice to T-lymphoblastic lymphoma. While a mutation in *Ikaros* gene lead to rapid development of leukemia and lymphoma [6]. BCL6 promotes the development of lymphomas in the mouse [7]. Oncogenic viruses were found to be associated with many established lymphoma cell line Burkett lymphoma cell line named katata found positive to human herpes virus-6 [8], another lymphoma cell line harboring Kaposi s sarcoma associated herpesvirus (KSHV/HHV-8) [9]. Epstein-Bar virus found with many established cell lines as B-cell lymphoma and T/NK-cell lymphoma [10,11]. Chemical carcinogens widely used to induce lymphoma. Nitrosoureas are used as positive control in carcinogenicity studies [12]. N-Methyl-N-nitrosourea one of its derivatives used to

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induce thymic lymphomas (MNU) [3]. N-butyl-N-nitrosourea also used to induce thymic lymphomas [14]. N-ethyl-N-nitrosourea that used in our research also induces thymic lymphomas [15,16]. [17] reported that cyclosporine promote murine lymphoid tumors induction.

Induction of tumors by chemical carcinogens in rodents is very important to study tumor biology and development of tumors [18]. The aim of the present work was to induce murine malignant lymphoma and establishment a long term culture to be used in lymphoma studies and anti-lymphoma drug development and to prove that nitrosourea is suitable carcinogen for lymphoma induction in local mice breeds.

Materials and methods:

Animals:

Sixty swiss Albino Male mice, 12 weeks old, weighing 20-25 g were used. They were provided with food and water ad libitum.

Chemical carcinogen

N-ethyl-N-nitrosourea (ENU, Bristol Laboratories, Italy) was used to initiate the carcinogenesis process. Cyclosporin-A (novartis - Swiss) was used as promoter.

Experimental design

Male mice were divided randomly into three groups (20 mice each), one control (untreated), group 2 which received during the first 2 weeks of the experiment, four i.p. injections of ENU (two injections/ week) at dose of 60 mg/kg (total of 240 mg/kg). Group 3 received at the first 2 weeks four i.p. injections of ENU (two injections/week) at dose of 60 mg/kg, then 15 mg/kg cyclosporine two times/week until the end of the experiment. They were kept on basal diet and water ad libitum until sacrifice at the 20^{th} weeks of experiment, when they were killed; complete gross examination was performed for detection of tumor masses. The thymus, spleen, liver, lung and kidney were collected for histopathological examination.

Culture of malignant lymphoma line *in vitro*.

When lymphomas were shown to be evident in mice, or the animals were very emaciated, the tumor was removed under sterile conditions. Tumor cultured by explanation method where cell suspensions were made in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics, by mincing the tissues and passing the crude suspensions through mish. After two washes, cell suspensions containing at least 5 X 10^6 cells per ml were seeded in 5 ml of medium in 25 cm^2 plastic culture flask (nunc, Denmark), and incubated at 37C°.

Cultures were fed daily by the addition of 1 ml of medium. Periodically part of the spent medium was replaced without disturbing the cultures. It was usually possible to begin subculturing at high cell densities about 2 weeks after initial explantation in vitro. After approximately 1 month, the cells were considered fully established. They were then passaged every 5-7 days by transferring small aliquots of cells from dens cultures into new flasks containing fresh medium.

Cytological studies:

Slide smear prepared by dropping sample from flasks on glass slid, fixed with absolute methanol, stained with crystal violet and examined by light microscope.

Karyotypic Analysis

Confluent monolayers of the cell line were treated with colchicine (0.03 mg/mL) for 2 hours and then dispersed with 0.5% trypsin. After having been washed with a physiological solution, the cells were treated with a 0.075 M KCl solution for 30 minutes at 37˚C. The cells were then fixed in a mixture of acetic acid and methanol (1:3, v/v) the cell suspension was dropped on a glass slide and stained [19].

Results

Tumor induction

Two emaciated mice out of 20 mice from the group 2 that received only ENU developed lung lymphoma which replaced the left lung completely, within 7 months after beginning of the treatment, Histopathological examination showed malignant lymphoma which replaced all the normal lung tissue in left lobe while there is diffuse infiltration of lymphoma in the right lobe. One mouse showed enlarged lymph nodes and developed a tumor in the cervical region with the same diagnosis figure(1). In the gross examination the spleen was enlarged in most of the treated mice figure(2). Surprisingly the second group which received cyclosporine until the end didn't develop any kind of tumors, but the mortality rate was high (15 out of 20 mice were dead at the time of the end of the experiment).

Fig(1): Swiss Albino mice from group 2 showing enlarged lymphatic area

Fig(2): Spleen of the induced mice 3 times bigger than the normal spleen

Cell culture

Cells began to divide and proliferate a few days after explanation as seen by the presence of mitotic figures in smears. The cells grow in clusters figure (3). The first subculture was made one week after explanation. By then cells were in continuous proliferation were the clusters enlarged in size and numbers figure (4). Single cells and clumps were present in the suspension culture with round well characterized cells figure (5). The population doubling time was (4-5) days during the early period following explanation, and later it stabilized at 48 hours. Single cells and clumps were present in the suspension cultures and there were several clusters distributed in the flask figure (6).

Cytological and light microscope examination

The cells were round in shape and had a tendency to make floating clusters. The cells had a smooth surface or protrusion on the margin of the cytoplasm, and proliferate in floatation and made large clusters as in figures (7), after passage 4 the cells start to show attachment and proliferate in to form monolayer figure (8).

Cytogenetic analysis:

The chromosome number was established from 20 counted cells. Of these, 12 cells contained 47 chromosomes.

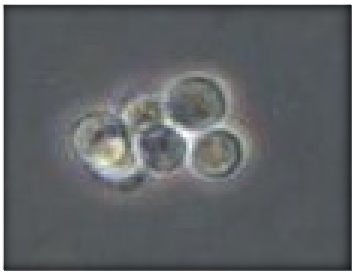

Fig (3): Cluster of lymphoblast cells in early primary culture.

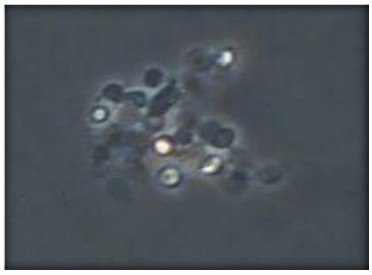

Fig (4): Cluster of lymphoblast cells after few days from primary culture.200x

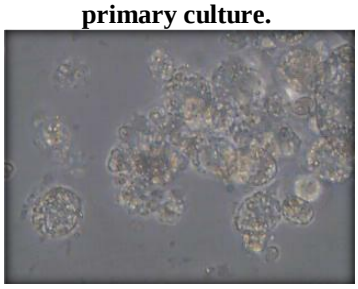

Fig (5): Cluster of lymphoblast cells in primary culture showed large round cells.400x

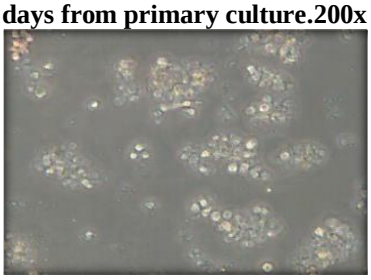

Fig (6): several cluster of proliferated lymphoma cells in passage 4, 100x

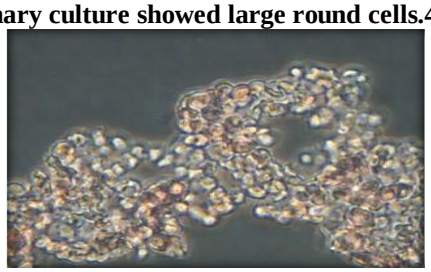

Fig (7): cluster tend to form large aggregations in passage 4, 200x

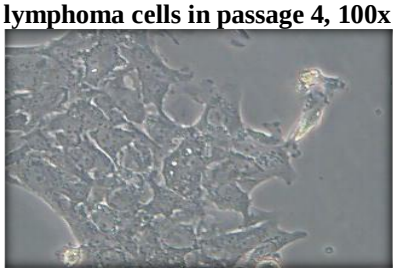

Fig (8): cells start to proliferate as attached cells to form a monolayer at passage 5. 200x

Discussion

Two emaciated mice of 20 mice from the group that received only ENU developed lymphoma, ENU is known to cause malignant lymphoma which used by many researcher for lymphoma induction [15,16,20]. The genetic mechanism for the development and progression of a lymphoma is unclear [21]. The tumor appear only in the group which administered ENU only, while the other group which administered also cyclosporine as a promoter didn't show any signs of lymphoma this disagree with [17] that reported cyclosporine promote murine lymphoid tumors induction. The tumor location of our lymphoma in lung tissue was reported in human only with plasmaplastic lymphoma as a unique case [22]. [23] explained mouse lymphoma infiltration to solid organs by using fibronectin receptor. This explains infiltration and replacement of entire lung tissue in our experiment. Furthermore, there was a numerical chromosomal abnormality in the lymphoma cells of diploid and polyploidy nature which is described as one of the continuous cell line properties [24]. In [21] study investigated the alterations in the DNA copy number and the expression profiles of the genes located in the altered regions in mouse thymic lymphomas that were induced by N-methyl-N-nitrosourea (MNU). The copy number gains of chromosomes 10 and 14 were observed in the MNU-induced lymphomas. The cancer-related genes

Pten, Top3b, and Ikaros were downregulated in lymphoma group while c-Myc was upregulated. The chromosome aberrations and novel expression profiles of the cancer-related genes within the altered regions may provide important clues to the genetic mechanism for the development of lymphoma [21].

The present malignant murine lymphoma cell line is established to simulate human malignant lymphoma, [26] refer to mouse models of human disease as a central part of many types of biomedical research, including cancer research. This because the laboratory mouse provides the most experimentally accessible mammalian model-one that shares organ systems, systemic physiology and genes with human [25]. Also it is very important tool for antitumor drug testing in cytotoxicity assay, which allow careful control of the physio-chemical and physiological environment and they give consistency of sampling and reproducible data where characterized cell strains are employed. This simplified statistical analysis and reduce number of replicates required. The drug concentration and the duration of exposure can be controlled more accurately than *in vivo* [26]. The present study aimed to establish a new *invitro* and *invivo* tumor models for cancer biology study and developing anticancer drugs, as a conclusion, nitrosourea is a suitable carcinogen for lymphoma induction which is recommended for future carcinogenesis studies.

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