Response of a multidrug-resistant human small-cell lung cancer xenograft to chemotherapy

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Abstract. Small-cell lung carcinomas (SCLC) are highly responsive to various chemotherapies. However only a minority of patients benefit from long survival. SCLC patients treated at the Institut Gustave Roussy received a combined chemotherapy (CCAV) including cisplatin, cyclophosphamide (Cpa), Adriamycin (doxorubicin; Adm) and vepeside (VP 16). We report here the intrinsic sensitivity of a small-cell lung carcinoma, designated SCLC-6, grafted in nude mice. This xenografted tumour was derived from an untreated patient. The CCAV regimen given to the patient donor of the tumour sample resulted in a complete response followed by recurrence and death, 8 months after the initial cure. The expression of P-glycoprotein encoded by the MDR1 gene was detected with the C219 antibody on the membrane of SCLC-6 tumour cells. When given to SCLC-6-tumour-bearing nude mice, CCAV induced a strong inhibition of tumour growth (84% of growth inhibition, 20 days after start of the treatment), but no cure. Intensification of CCAV doses did not improve the response. The efficacy of individual agents of the CCAV, given at maximal tolerated doses was analysed. Only cisplatin (10 mg/kg) and Cpa (3×50 mg/kg) inhibited SCLC-6 growth (79% and 100% inhibition respectively), VP16 (3×24 mg/kg) was poorly efficient (42%) and Adm (10 mg/kg) not at all. Two-drug combinations such as cisplatin plus VP16 or cisplatin plus Cpa inhibited tumour growth (81% and 70%, respectively). Curiously, the efficacy of Cpa, given in combination with cisplatin was less than that of Cpa alone. Repeated treatments with CCAV administered to mice at each in vivo passage of the tumour induced a loss of chemosensitivity, which was observed until the ninth passage. An improvement of the therapeutic response was obtained by adding a headline reverser of multidrug resistance, verapamil (25 mg/kg), to CCAV (81% versus 63% inhibition). MDR1-related resistance appeared to play a role in the failure of SCLC-6 chemotherapy; frequent recurrences after treatment with cisplatin and Cpa, two drugs that are not recognized by the P-glycoprotein, indicated that other modes of resistance were simultaneously active.

Key words: Small-cell lung cancer – Xenograft – Chemotherapy – P-glycoprotein – Multidrug resistance

Introduction

Escape from chemotherapy is a major problem in the treatment of many malignancies. Tumours such as small-cell lung cancers (SCLC), which are initially responsive to chemotherapy, can develop resistance during treatment with cytotoxic agents. Clinically this is characterized by short periods of remission and failure to respond to subsequent therapy (Houghton et al. 1985). Combination chemotherapy has significantly improved the response rates for a variety of human tumours. A combination of several drugs may be more effective than the best single agent for four main reasons: (a) because separate agents have different limiting toxicities and can therefore be combined at doses close to their maximum single-agent levels; (b) because one agent can fail to reach disease confined to an organ, as if the tumour were in a pharmacological sanctuary; (c) because a degree of potentiation exists between the agents in their efficacy against tumour cells to a greater extent than against normal cells (Stephens et al. 1977); (d) finally because different cellular mechanisms of resistance can be activated by different agents. In an investigation of a series of 20 tumour lines, obtained by transplantation into nude mice of human SCLC samples direct from patients, we (Arvelo et al. 1990) reported that 5 (25%) overexpressed the P-glycoprotein. This protein is detectable by antibodies at the cell membrane of resistant cells (George et al. 1990), actively excludes numerous antitumoral agents from the cell cytoplasm, and confers a multidrug-resistance phenotype to the cells. Holzmayer et al. 1992 published a correlation between clinical effects and overexpression of P glycoprotein in SCLC. In the present study, therefore, using
the SCLC-6 tumour line, a SCLC line growing as xenografts in nude mice overexpressing the P-glycoprotein, we investigated its response to various agents and regimens, usually used in chemotherapy.

Materials and methods

Nude mice: Swiss (nu/nu) female mice, 6-8 weeks old, were purchased from IFFA-Credo, Lyons, France. The animals were maintained in specific-pathogen-free conditions.

Tumour. The SCLC-6 tumour was established directly as a xenograft from a metastatic suprachlavicular lymph node resected in an untreated 44-year-old man, a smoker, with a small-cell lung carcinoma. The patient then received a combination regimen (CCAV) including cyclophosphamide (Cpa), cisplatin, Adriamycin (Adr; doxorubicin) vesepside (VP16) and irradiation. The patient achieved complete remission after six cycles of the CCAV regimen. A relapse caused death, 8 months later. Histologically, the human biopsy specimen and its corresponding xenograft fulfilled WHO criteria for an intermediate subtype of SCLC. Cytogenetic studies confirmed the human origin of the xenografted tumours. Tumours were serially transplanted from mouse-to-mouse as follows: tumour-bearing mice were sacrificed after over-anesthesia and tumours were excised. Tumoural tissue was cut into pieces of 2-3 mm. Recipient mice were anaesthetized using ether, a short skin incision was made in the interscapular area, a piece of tumour was inserted into the sub-cut tissue and the skin was closed with one agraffe.

Evaluation of the therapeutic effect of the different treatments. When the tumours reached a mean diameter of 5×10 mm, the tumour-bearing mice were randomly divided into groups of 5-15 animals each and treatment with drugs was started. All agents were injected by the i.p. route. Each drug was given at the maximal dose tolerated by the nude mouse. Because there is no universally satisfactory basis for converting human doses to the corresponding murine doses, the policy that we adopted was to define the maximal tolerated dose of each agent as determined from preliminary toxicity studies, based on the loss of weight (exceeding 10%) or death; 10 mg/kg Adm (Roger Bellon, France); 3x24 mg/kg VP16 (Sandoz, France); 10 mg/kg cisplatin (Lilly, France); and 3x50 mg/kg Cpa (Lucien, Colombes, France). When the four drugs were combined (CCAV) doses of each agent were adjusted to those reported in Table 1: 6 mg Adm, 3 x 8 mg VP16, 3 mg/kg cisplatin and 3x50 mg/kg Cpa. In each experiment, a control group of mice received a daily injection of isotonic NaCl solution. All agents were injected in a volume of 0.02 ml/kg body weight. Daily hydration of mice was achieved by injecting 0.3-0.5 ml 5% glucose solution twice per day during CCAV administration and for 2 additional days. Mice were sacrificed by over-anesthesia when the tumour reached a volume of 4 cm³. The tumour growth was followed by measuring two diameters every 2-3 days with a caliper. The tumour volume was calculated as a function of an ellipsoid volume by the formula \( V = \pi ab^2/2 \), where \( a \) is the tumoral width and \( b \) is the length in millimeters (Geran et al. 1972). The tumour sizes were standardized in different groups by using relative tumour volumes \( (V_x) \) calculated by the formula \( V_x = V/V_0 \), where \( V_x \) is the mean tumour volume at any given time and \( V_0 \) is the mean initial tumour volume at the start of treatment (approximately 250 mm³). Statistical analysis of the differences between the mean volume was carried out according to Student’s t-test, comparing the \( V_x \) values at a given time. The effect of drugs was expressed as the percentage inhibition as a T/C ratio [mean size of the treated tumour/mean size of tumour in control group]×100.

Establishment of SCLC-6 cell line. The SCLC-6 cell line was established in short-term culture from SCLC-6 tumour tissue, after heterotransplantation into nude mice (after the third to the tenth passage). A piece of tumour tissue was collected under aseptic conditions. Immediately after removal, the tumour specimen was placed in plastic petri dishes containing phosphate-buffer saline (PBS) plus 100 µg/ml penicillin, and 100 µg/ml streptomycin. Tumours tissue was separated mechanically from macroscopically recognizable connective tissue. The tumour was minced using sterilized scissors and tweezers. A mechanical method was used to achieve further disintegration of tumour fragments. This method consisted of passing the tumour fragments, resuspended in EDTA (ethylenediaminetetraacetic acid)/PBS 0.02%, successively through 20-, 21- and 23-gauge steel mesh and then vigorous trituration with a pipette. The resulting suspension was washed twice in PBS, then set up in culture in a RPMI-1640 medium supplemented with 10% fetal calf serum plus antibiotics.

Monoclonal antibody. The murine monoclonal antibody C219 (ORIS, IgG2a) coupled to fluorescein isothiocyanate (FITC), specific for P-glycoprotein, was used for direct immunofluorescence staining. It recognizes a highly conserved cytoplasmic epitope found in all P-glycoprotein expression (Georges et al. 1990).

Simultaneous analysis of P-glycoprotein expression and cell cycle. SCLC-6 cells were harvested and washed twice in PBS (pH 7.2) without Ca²⁺ and Mg²⁺. A cell suspension of 10⁶ cells/ml was treated with the FITC-conjugated C219 antibody diluted 1:20 in PBS containing 1% bovine serum albumin (BSA). An identical cell suspension was treated with a murine FITC-conjugated IgG2a antibody, used at the same dilution to determine non-specific binding. After 30 min incubation in the dark at 4 °C, the cells were washed twice in 1% BSA/PBS (pH 7.2). The fluorescein-stained cells were then treated with 2.5 mg/ml RNase (Sigma) for 30 min at 37 °C to remove RNA, which would affect DNA analysis. The cells were then centrifuged and the cell pellet was treated with 4 µg/ml propidium iodide (Sigma) for 30 min at 4 °C in the dark. The cells were adjusted to 10⁶ cells/ml for flow-cytometry analysis.

Flow-cytometry analysis. Flow-cytometry analyses were performed on a FACSCan flow cytometer (Becton Dickinson, Mountain View, Calif.). The excitation source was an argon ion laser emitting a 488-nm beam at 15 mW. The green fluorescence of FITC (\( \lambda_{Em} = 494 \) nm, \( \lambda_{Ex} = 517 \) nm) was collected through a 520/30-nm band-pass filter, while the red fluorescence of propidium iodide (\( \lambda_{Em} = 540 \) nm, \( \lambda_{Ex} = 625 \) nm) was collected through a 585/42-nm band-pass filter. Samples containing 10⁶ cells were analysed. The green fluorescence, related to P-glycoprotein expression, was measured on a logarithmic scale and the red fluorescence, related to DNA content, on a linear scale. The data were analysed with the logical LYSYS program (Becton Dickinson, Mountain View, Calif.).

Results

In order to investigate the therapeutic effect of the different treatments, we used the SCLC-6 tumour transplanted into nude mice. The doubling time of SCLC-6 was 4 days (mean). The various therapeutic regimens are detailed in Table 1, which also reports the results obtained with the different treatments on SCLC-6 xenograft growth.

Single-course treatment with the CCAV combination

Administration of these four drugs constituting the CCAV was sequential (see details in Table 1). On the first day, tumour-bearing mice received Adm and VP16; day 2, VP16 and cisplatin; day 3, VP16 and Cpa; on days 4 and 5 Cpa was administered alone. Out of 15 mice, 14 responded to chemotherapy, with a mean tumour volume inhibition of 84%, compared to the mean tumour volume of the control group (P<0.01), when growth inhibition was evaluated between day 10 and day 20 (Table 1). Inhibition of tumour growth was noticeable at day 5, and reached a plateau as early as day 9. Despite this significant response to therapy, the tumours