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Relationship of LRP-human major vault protein to *in vitro* and clinical resistance to anticancer drugs

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Abstract

Multidrug resistance (MDR) has been related to two members of the ABC-superfamily of transporters, P-glycoprotein (Pgp) and Multidrug Resistance-associated Protein (MRP). We have described a 110 kD protein termed the Lung Resistance-related Protein (LRP) that is overexpressed in several non-Pgp MDR cell lines of different histogenetic origin. Reversal of MDR parallels a decrease in LRP expression. In a panel of 61 cancer cell lines which have not been subjected to laboratory drug selection, LRP was a superior predictor for *in vitro* resistance to MDR-related drugs when compared to Pgp and MRP, and LRP's predictive value extended to MDR unrelated drugs, such as platinum compounds. LRP is widely distributed in clinical cancer specimens, but the frequency of LRP expression inversely correlates with the known chemosensitivity of different tumour types. Furthermore, LRP expression at diagnosis has been shown to be a strong and independent prognostic factor for response to chemotherapy and outcome in acute myeloid leukemia and ovarian carcinoma (platinum-based treatment) patients. Recently, LRP has been identified as the human major protein. Vaults are novel cellular organelles broadly distributed and highly conserved among diverse eukaryotic cells, suggesting that they play a role in fundamental cell processes. Vaults localise to nuclear pore complexes and may be the central plug of the nuclear pore complexes. Vaults structure and localisation support a transport function for this particle which could involve a variety of substrates. Vaults may therefore play a role in drug resistance by regulating the nucleocytoplasmic transport of drugs.

Abbreviations: LRP – Lung Resistance-related Protein; MVP – Major Vault Protein; MDR – Multidrug resistance; MRP – Multidrug resistance-associated Protein; NPC – Nuclear Pore Complex, Pgp – P-glycoprotein.

Introduction

Broad resistance to chemotherapeutic drugs is a major cause of failure of cancer treatment. *In vitro* this phenomenon can occur as a result of exposure of cancer cells to a single cytotoxic drug and is termed multidrug resistance (MDR) (Childs and Ling, 1994). MDR has been associated with the overexpression of P-glycoprotein (Pgp) or Multidrug Resistance-associated Protein (MRP) (Childs and Ling, 1994). However, there is increasing evidence from *in vitro* and clinical studies that additional mechanisms of MDR may be operative. A novel protein associated with MDR, originally termed the Lung Resistance-related Protein (LRP), has been described (Scheper, 1993). This article reviews the data supporting the association of LRP with drug resistance *in vitro*, its distribution in normal human tissues, as well as its potential value as a marker

of clinical drug resistance. In additional, the molecular characterization of LRP and the possibility that LRP may be involved in an entirely new mechanism of drug resistance is discussed.

Identification of the Lung Resistance-related Protein (LRP) in SW-1573 non-small-cell lung cancer MDR sublines

A series of MDR sublines was developed from the SW-1573 non-small cell lung cancer cell line by exposure to increasing concentrations of doxorubicin. The 2R120 subline showed moderate levels of resistance to doxorubicin, vincristine, and etoposide (4- to 45-fold), in the absence of Pgp expression (Kuiper, 1990). The 2R120 cell line was chosen to investigate non-Pgp-mediated MDR. BALB/c mice were immunized with 2R120 cells and the monoclonal antibody LRP-56 was selected for strong immunoreactivity with 2R120 cells compared to parental SW-1573 cells (Scheper, 1993). LRP-56 specifically reacted with a 110 kDa protein, LRP, which was overexpressed in 2R120 cells. The SW-1573/2R120 revertant cell line (2R120 cell line cultured without drug for over nine months) showed a decrease in the level of resistance to approximately equal levels to the parental cells. Of interest, this cell line also showed a decrease in the level of LRP expression, further supporting a close association between LRP and drug resistance in 2R120 celis. Furthermore, the 2R160 cell line, a SW-1573 subline displaying high levels of risistance and increased MDR1/Pgp expression had very low LRP expression, similar to the parental SW-1573 cells (Scheper, 1993).

LRP-56 displays a characteristic cytoplasmic punctate staining pattern in 2R120 cells (Scheper, 1993). This staining pattern is conserved in other MDR cell lines, in normal human tissues, and in human malignancies.

LRP overexpression in MDR cancer cell lines

The overexpression of LRP in the 2R120 cell line was found not to be a peculiarity of these cells, but a more general feature of non-Pgp-mediated MDR. LRP has also been found to be overexpressed in a number of other Pgp-negative MDR cell lines of different histogenetic origin (Scheper, 1993). These include cell lines derived from small cell lung cancer (GLC4/ADR), fibrosarcoma (HT1080/DR4), breast cancer (MCF7/Mitox), and myeloma (8226/MR40). The up-regulation of LRP occurs early during the process of drug selection in various series of MDR sublines, such as those derived from the SW-1573, and GLC4 cells (Scheper, 1993; Versantvoort, 1995). This observation suggests that the LRP-associated mechanism is already involved in low or moderate levels of drug resistance, which are likely to be more clinically relevant.

Most LRP overexpressing MDR cell lines also display increased levels of MRP (Scheper, 1993; Flens, 1994). Although these two MDR-related proteins are frequently co-upregulated in MDR cell lines, there is evidence that both genes can be regulated independently (see below). Since transfection of the MRP gene itself has been associated with low or moderate levels of drug resistance (Zaman, 1994), the concomitant operation of several drug resistance mechanisms may be necessary to achieve the high levels of resistance observed in most LRP and MRP positive MDR cells. In contrast, most Pgp-positive MDR cell lines do not overexpress LRP, like the non-small cell lung cancer SW-1573/2R160, the ovarian carcinoma A2780AD, and the myeloma 8226/Dox 6 and 8226/Dox40 cell lines (Scheper, 1993). However, LRP and Pgp expression are not always mutally exclusive. The MCF7/D40 breast cancer cell line and certain 8226 myeloma sublines showed increased levels of both Pgp and LRP (Scheper, 1993; Shao, 1995). Despite the similar Pgp content observed in independently isolated 8226 MDR sublines, the resistance levels were substanstially higher in a Pgp/LRP positive 8226 subline than in the Pgp positive/LRP negative 8226/Dox 6 subline, suggesting that LRP has functional relevance in certain Pgp overexpressing MDR cells (Shao, 1995). Remarkably, LRP overexpression has been reported in Pgp/MRP negative MDR cell lines, such as the mitoxantrone selected MCF7/MR cell line (Futscher, 1994). In these cells, the LRP-associated mechanisms of MDR may play a prominent role.

Relation of LRP to drug resistance in human cancer cell lines not celected in the laboratory for drug resistance

Studies on the mechanisms of resistance have concentrated on laboratory selected MDR cancer cells generated through a stringent drug-treatment selection procedure, and in general, show (very) high levels of resistance. Therefore, it may be difficult to extrapolate the

results obtained to the clinic. Cancer cell lines not subjected to laboratory drug selection may more accurately reflect the characteristics of the tumors from which they have been derived. The relation of LRP, as well as of Pgp and MRP, to drug resistance was studied in a panel of 61 human cancer cell lines used at the National Cancer Institute (USA) for screening of new anticancer drugs. Using immunocytochemistry, LRP and MRP were expressed in 78% and 87% of the cell lines, respectively, suggesting that the LRP- and MRP-associated resistance mechanisms are widespread in human malignancies (Izquierdo, 1995a). In contrast, Pgp was detected at relatively low levels in 24% of the cell lines. Among the three MDR-associated proteins, LRP showed the greatest individual value as a marker of *in vitro* resistance to a variety of MDR related drugs (i.e doxorubicin, vincristine), which also applied to non-classical MDR drugs (i.e. cisplatin, carboplatin and melphalan) (Izquierdo, 1995a). The LRP associated phenotype of drug resistance seems to be broad, including drugs which are not substrates for Pgp or MRP. This may indicate that LRP is frequently co-expressed with other resistance mechanisms. However, a direct role for the LRP-associated mechanism in resistance to some non-classical MDR drugs, such as carboplatin, deserves further investigation.

Expression of LRP in normal human tissues and tumours

Proteins related to drug resistance *in vitro* have been demonstrated in various normal human tissues and their malignant counterparts. In normal cells, they appear to play a protective role against toxic compounds. Evidence suggests that the cells retain this function also in the malignant phenotype and thus, neutralize the cytotoxic effects of anticancer drugs. LRP has been found to be widely distributed in human normal tissues and tumors (Izquierdo, 1996; Scheper, 1993). However, distinct patterns of expression were noticed. High LRP expression was seen in tissues chronically exposed to xenobiotics (i.e. bronchus, digestive tract, and keratinocytes), in metabolically active tissues (i.e. adrenal cortex), and in macrophages, whereas varying levels were observed in other organs (Izquierdo, 1996). This distribution resembles that of other drug resistance associated proteins such as Pgp and MRP (Flens, 1996), and suggests a role for LRP in defense against xenobiotics. Among 174 tumour specimens comprising 27 tumour types, LRP was expressed in 63% of the cases. This figure was consistent with the *in vitro* data indicating the widespread distribution of the LRP-associated mechanism of drug resistance in human malignancies. It was of interest to note that the distribution of LRP was not uniform among different tumour types. Rather, LRP expression closely reflected the susceptibility to chemotherapy of different tumour types. Highly chemosensitive cancers (i.e. germ cell tumors, leukemias, neuroblastoma) expressed LRP in a minority of cases, partially chemosensitive cancers (i.e. ovarian carcinoma) in a large proportion of cases, and refractory cancers to chemotherapy (i.e. colon, renal, and pancreatic cancers) were LRP positive in the vast majority of instances (Izquierdo, 1996). These results further support the potential value of LRP as a marker of clinical resistance to chemotherapy.

The immunohistochemical expression of Pgp, MRP and LRP was studied in 21 primary and 37 metastatic malignant melanoma specimens (Schadendorf, 1995). Pgp was detected in only one case and MRP in 50% of the tumors (with no difference between metastatic specimens taken prior to or after chemotherapy). LRP was expressed in 13/21 and 23/37 primary and metastatic tumors, respectively. The majority of metastatic specimens that expressed LRP in more than 50% of tumor cells had been previously exposed to chemotherapy. The significant expression of MRP and LRP provides insight into the drug resistance phenotype in malignant melanoma.

Clinical value of LRP to predict response to chemotherapy and prognoses

To date, three studies have investigated whether the expression of LRP in clinical cancer specimens is predictive of response to chemotherapy and prognoses. In one study including 30 patients with relapsed childhood acute lymphoblastic leukemia, the expression of LRP, but not of Pgp, was significatively associated with an increased *in vitro* resistance of fresh leukemic cells to daunorubicin (Klumper, 1995). In a prospective study including 87 patients with adult acute myeloid leukemia, LRP was found to be an independent prognostic variable for response to natural products based chemotherapy (36% and 56% response rates in LRP positive and LRP negative patients, respectively), and for progression-free survival, and the prognostic value of LRP was superior to that of Pgp (List, 1993). Similarly, the expression of LRP in the tumours of 57 women with FIGO stage III/IV ovarian cancer indi-

cated that LRP positive tumours had a significantly inferior response to platinum and alkylating based chemotherapy (8% complete, 36% partial, and 56% no response) as compared to LRP negative tumours (50% complete, 30% partial, and 20% no response) (Izquierdo, 1995b). Furthermore, the expression of LRP was significantly associated with a shorter interval until tumour progression and shorter overall survival. The fact that LRP shows strong prognostic value in different cancers treated with dissimilar chemotherapeutic regimens is certainly remarkable. These results agree with the capacity of LRP to predict a broad resistance phenotype in the NCI panel, which is composed of eight different cancer types (Izquierdo, 1995a).

Chromosome localisation of the LRP gene on chromosome 16

Using fluorescence *in situ* hybridization, the LRP gene has been localised to the short arm of chromosome 16, within the 16p 13-1-16p 11.2 chromosomal region (Scheffer, 1995; Slovak, 1995). Two other MDR-associated genes have als been mapped to this region: the MRP gene and the protein kinase C-β gene (involved in MDR by increasing the transport activity of Pgp upon phosporylation). This chromosomal region may be critically involved in cellular defense against cytotoxic agents, including anticancer drugs. Despite the frequent co-upregulation and close chromosomal localisation of the LRP and MRP genes, it is evident that each can be switched on separately and do not belong to the same amplicon. Cancer cell lines and clinical trumour specimens exist that express only one of these two proteins (Izquierdo, 1995a; 1995b). In addition, it has been reported that H69AR cells contain amplified MRP gene sequences only in homogeneously staining regions (HSR) and double minutes, whereas HT1080/DR4 cells contain amplification of both LRP and MRP in a striking striped pattern in an HSR (Slovak, 1995).

Molecular characterization of LRP as the human major vault protein

The molecular characterization of LRP has constituted the first step to clarify its functional significance in normal and malignant human tissues, as well as its role in drug resistance. A cDNA coding for the LRP gene was isolated from the human fibrosarcoma cell line HT1080/DR4 (Scheffer, 1995). Comparative sequence analysis indicated that LRP shares 57% and 87.7% amino acid identity with the major vault protein from *Dictyostelium discoideum* and *Rattus norvigecus*, respectively (Kickhoefer, 1994; Vasu 1993). Thus, LRP was identified as the human major vault protein (MVP), which is the most abundant component of previously described multisubunit particles termed vaults (Kedersha, 1986; Rome, 1991).

Vaults: cellular organelles in search of a function

Vaults were first identified by negative staining and transmission electron microscopy in 1986, as contaminant particles of clathrin-coated vesicle preparations derived from rat liver (Kedersha, 1986; reviewed in Rome, 1991). Subsequently, vaults have been isolated from various species including the lower eukaryote *D. discoideum*, amphibians (frog), avians (chicken), and mammals (Rat, rabbit, cow). In agreement with LRP distribution in normal human tissues, vaults are most abundant in epithelial cells (i.e. rat intestine) and macrophages (i.e. rabbit alveolar macrophages) in other species (Kedersha, 1990). despite the complex composition and structure of vaults, they are highly conserved among phylogenetically dissimilar species, supporting the notion that their function is essential to eukaryotic cells (Kedersha, 1990). Vaults are ribonucleoprotein particles which, in the rat, are composed of a MVP of 104 kD (accounting for > 70% of the mass of the particle), three minor proteins of 210, 192, and 54 kD, and a small RNA molecule. Antibodies raised against rat vaults recognize the MVP in all eukaryotic species tested, including, *Drosophila*, dog, and humans. The vault components are assembled in a barrel-like structure of $\sim 57 \times 32$ nm with a molecular mass of about 13 MDa, composing the largest ribonucleoprotein body reported to data (three times the size of a ribosome) (Figure). The vault particle has 2-fold symmetry, and each half can be opened into a flower-like structure which contains eight petals surrounding a central ring (Kedersha, 1991). These dynamic structural variations are likely to play a role in vault function, Most vaults are present in the cytoplasm and most cells contain thousands of vaults. To date, the precise function of vaults is unknown. A small fraction of vaults are localised to the nuclear membrane and nuclear pore complexes (NPC) (Chugani, 1993). This localisation and structural similarities support the hypothesis that vaults constitute the central plugs of the NPC. This

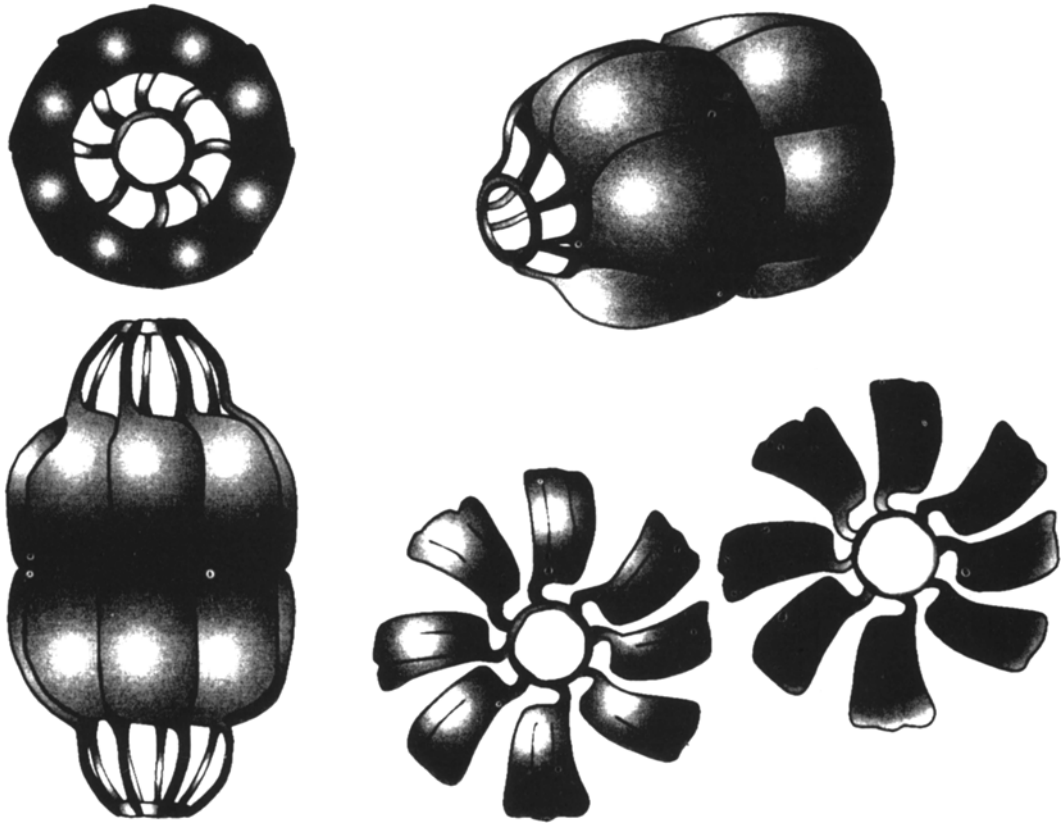

Figure 1. A model of vault structure. An intact vault particle is illustrated in end-on, oblique and side views (to and lower left) and in the unfolded dual-flower conformation (lower right).

raises the possibility that vaults mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm (Chugani, 1993).

Vaults in drug resistance: functional hypothesis

Studies using laser-assisted confocal microscopy have shown that MDR cancer cells display an altered intracellular distribution of drugs as compared to parental cells. MDR cells distribute daunorubicin into the perinuclear region and, subsequently, redistribute the drug away from the nucleus into a punctate cytoplasmic pattern, whereas parental cells localise daunorubicin in a diffuse nuclear and cytoplasmic pattern (Gervasoni, 1991). Similarly, reduced nuclear accumulation of daunorubicin has been reported in the LRP overexpressing MDR cell line 2R120 (Schuurhuis, 1991). The perinuclear and cytoplasmic structures to which daunorubicin is distributed in MDR cells are unknown. Vaults appear to be good canditates. Considering the large size and abundance of vaults in the cytoplasm, the punctate staining pattern of LRP-56, as well as the evidence supporting vaults as plugs of NPCs, it is tempting to hypothesise that vaults may play a role in drug resistance by regulating both the cytoplasmic redistribution and the nucleocytoplasmic transport of drugs. Transfection of the LRP gene alone has failed to confer MDR, an expected finding considering that the complete vault particle will be required for functional activity (Scheffer, 1995). Ribozyme and antisense approaches aimed at disruption of vaults are under investigation to clarify the functional role of vaults in drug resistance.

Concluding remarks

The data obtained so far indicate that LRP, the human MVP, is frequently expressed in normal and malignant

cells suggesting a fundamental biological function for vaults. The clinico-pathological studies performed in human malignancies indicate that the expression of LRP is associated with a poor response to chemotherapy and adverse prognosis. Additional studies in various tumour types are certainly warranted to fully elucidate the role of LRP as a clinical drug resistance marker. The ongoing functional characterization of vaults will clarify its actual role in a potential new mechanism of drug resistance and may reveal innovative approaches to prevent the emergence of resistance or reverse the phenotype when it occurs.

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