survival rates to > 50% over the period of 1992 – 1997 [3]. Data from Europe have demonstrated increases in 5-year survival that vary from 26% for Eastern Europe to 42% for Northern Europe [4]. However, the prognosis for patients with ovarian cancer remains poor. Up to 75% of patients are diagnosed in the advanced stage and many require chemotherapy after cytoreductive surgery [5]. Although 10 – 15% of patients maintain a response to standard first-line cisplatin/paclitaxel chemotherapy, most patients eventually relapse [6]. The goals of treating advanced recurrent ovarian cancer are mainly palliative, attempting to prolong life and control disease-related symptoms, while minimising treatment-related toxicities and maximising health-relapsed quality of life.

Some significant advances in clinical oncology using standard- or high-dose regimens have been achieved, but such gains seem to have reached a plateau over the past two decades, in part as a result of drug resistance. The shift to alternative targets within the tumour and the use of these targets for the subset of patients who, either because of intrinsic or acquired resistance, are not likely to respond to standard therapy holds promise. The results of Colleoni *et al.* [7] may herald a gradual shift from standard maximum tolerated dose (MTD) or high-dose chemotherapy, to, at least in the chemoresistant population, induction of antiangiogenesis by low-dose chemotherapy. At present, most of the new receptor blocking agents such as gefitinib (ZD-1839/Iressa®, AstraZeneca Pharmaceuticals LP) or cetuximab (C-225/Erbitux™, ImClone Systems Incorporated), as well as antiangiogenic drug (e.g., bevacizumab/Avastin™ [Genentech, Inc.]: the humanised monoclonal antibody to vascular endothelial growth factor [VEGF]), are used with standard chemotherapy regimens, which negates their superior safety profiles. As the cancer patient population ages, should these combinations also be evaluated in the setting of low-dose, frequent, continuous chemotherapy? The time may come when the term 'side effect' for chemotherapeutic drugs not only loses its negative connotations, but takes on a new, and positive, meaning.

2. Induction chemotherapy (primary chemotherapy)

Surgery followed by systemic chemotherapy is the current standard treatment modality for epithelial ovarian cancer, particularly when diagnosis is made at an advanced stage [8,9]. The combination of paclitaxel and cisplatin replaced schemes without paclitaxel after it was shown in the Gynecologic Oncology Group Trial 111 [10] and in a subsequent confirmatory trial [11] that it was more effective than the combination of cyclophosphamide and cisplatin. In fact, paclitaxel combined with carboplatin is considered the standard first-line chemotherapy regimen worldwide because of its more favourable toxicity profile as compared with paclitaxel and cisplatin [12-14]. Surgery and first-line systemic chemotherapy induce complete and partial response in ≤ 80% of patients, with a pathological complete remission rate of ~ 25% [10,11]. Unfortunately, recurrences occur in the majority of patients, and only 20 – 40% survive after a 5-year follow-up period, with survival being substantially dependant on the initial International Federation of Gynecology and Obstetrics stage [15].

Important questions about the clinical value of platinum/taxane combinations have been raised by the results of the large International Collaborative Ovarian Neoplasm Group 3 study involving 2074 ovarian cancer patients. The data from this trial suggest that there was no benefit, in terms of either progression-free or overall survival, from the use of paclitaxel/carboplatin compared with carboplatin alone or cyclophosphamide/doxorubicin/cisplatin [16]. Furthermore, the incidences of alopecia, fever and sensory neuropathy were significantly higher in the taxane treatment arm compared with carboplatin alone. The SCOTROC Randomised trial in Ovarian Cancer has compared the use of two different taxane preparations in combination with platinum to determine whether there were any differences in efficacy or tolerability. A total of 1077 patients were randomised to receive either docetaxel/carboplatin or paclitaxel/carboplatin [17]. The results indicate that there was no significant difference between these regimens in terms of either median progression-free survival (15.1 months for docetaxel/carboplatin versus 15.4 months for paclitaxel/carboplatin) or overall survival at 18 months (73.5 versus 76.6%, respectively). However, there were some differences between the two treatment groups regarding their tolerability profiles, with paclitaxel associated with significantly greater neurotoxicity, arthralgia/myalgia and weakness in the legs or arms compared with docetaxel. Nevertheless, global quality of life parameters based on the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 questionnaire were comparable in both treatment arms. These data suggest that individual patients might benefit from the use of one or the other taxane, depending on their predisposition to adverse effects such as neuropathy.

Recent studies assessing the effects of the addition of epirubicin to platinum/taxane have shown a higher response rate among patients in the epirubicin treatment arm compared with those receiving platinum/taxane alone, although there was also a higher incidence of toxicity in these patients [18]. A number of newer chemotherapeutic agents are being assessed for a potential role in first-line treatment regimens for ovarian cancer, including gemcitabine, pegylated liposomal doxorubicin, irinotecan, oxaliplatin and topotecan. Of these agents, topotecan has been extensively studied using a variety of different treatment strategies. The mechanism of action of topotecan (inhibition of topoisomerase I) is different from that of paclitaxel, with no overlap, and synergy has been demonstrated in *in vitro* tumour models with paclitaxel and platinum [19,20]. Topotecan has also shown activity in platinum- and paclitaxel-resistant tumours, and there is an absence of cross-resistance with paclitaxel [21]. Likewise, in Japan, irinotecan (but not topotecan) is frequently used for platinum- and paclitaxel-resistant tumours. Both clear cell carcinoma and mucinous cystadenocarcinoma in advanced stages are poorly responsive

to platinum- or taxane-based chemotherapy [22-24]. In addition, the authors of this review have examined response rates to standard regimens according to histological type. The response rate of clear cell carcinoma was significantly lower (showing 11.1%), compared with 72.5% of serous cystadenocarcinoma [22]. In patients with > 2 cm residual tumour the response rate to cyclophosphamide/adriamycin/cisplatin (CAP) regimen was also lower in mucinous cystadenocarcinoma and clear cell carcinoma compared with serous cystadenocarcinoma and endometrioid adenocarcinoma. However, when etoposide/cisplatin and irinotecan/cisplatin were used to treat mucinous cystadenocarcinoma and clear cell adenocarcinoma, respectively, significant response rates (33 and 50%, respectively) were obtained [25]. The standard regimen for clear cell adenocarcinoma and/or mucinous adenocarcinoma should be evaluated by independent trials. Thus, this group are using a standard regimen (paclitaxel/carboplatin) to treat serous cystadenocarcinoma and endometrioid adenocarcinoma as a first-line chemotherapy, whereas combination chemotherapy using etoposide and cisplatin to treat mucinous cystadenocarcinoma, and combination of irinotecan and cisplatin to treat clear cell carcinoma are used as a first-line chemotherapy.

3. Second-line chemotherapy (salvage, consolidation, maintenance chemotherapy)

Aggressive surgical cytoreduction followed by six cycles of carboplatin plus paclitaxel represents the standard of care for ovarian cancer, from stage 1C to IV [8,9,12-14]. Despite the high response rate reported with this strategy, most (50 – 75%) of the patients who have a complete response relapse ultimately die of ovarian cancer [15,26].

Several types of consolidation treatments have been tested, such as radiotherapy [27,28], hormonal therapy [29] and immunotherapy [30,31]. Most of these studies had small sample size and insufficient power; all of them produced negative results. Recently, two studies have been reported on the use of systemic chemotherapy as consolidation treatment with paclitaxel and epirubicin [32,33]. Markman *et al.* [32] showed that 12 cycles of single-agent paclitaxel, compared with 3 cycles of the same drug, significantly prolonged progression-free survival in patients with clinical complete response to first-line carboplatin and paclitaxel. This study was discontinued early after an interim analysis showed a statistically significant improvement in time to progression, with a 7-month advantage for the arm receiving 12 cycles compared with that receiving 3 cycles. This is the first randomised study that has suggested that maintenance chemotherapy may impact survival. In addition, it has been reported that chronic administration of single weekly paclitaxel in heavily pretreated ovarian cancer patients could be safely used and resulted in long progression-free interval [34].

Another trial with negative results has been reported in abstract form by Scarfone *et al.* [33], comparing four cycles of epirubicin (120 mg/m^2) with no treatment in the same setting of patients. Preliminary results (presented at the 2002 Annual Meeting of the American Society of Clinical Oncology) indicate that there was no advantage in time to progression for patients treated with epirubicin. The addition of epirubicine to the standard carboplatin and paclitaxel treatment did not improve progression-free survival [35,36].

Improvements in ovarian cancer management mean that it may now be a long-term disease for which treatment must be carefully considered. Optimal sequencing of chemotherapy may help to enhance patient's benefit of therapy and minimise toxicity. The response to retreatment with platinum or a platinum/taxane combination is strongly influenced by the treatment-free interval after initial therapy with a platinum combination. Response rates to platinum retreatment in platinum-resistant patients (relapse within 6 months) are lower than those in platinum-sensitive patients (relapse after 6 months). It is possible that if one was able to extend the interval until relapse, response rate to platinum may be improved. Therefore, increasing the platinum-free interval by using non-platinum-based chemotherapy for treatment after relapse appears to increase the response to later rechallenge with platinum [37]. Many alternative agents have been investigated for the treatment of patients with relapsed ovarian cancer. For the selection of the optimal chemotherapy regimen at first relapse, patients are usually characterised according to their degree of sensitivity or resistance to the treatment, depending on the interval between initial response and first relapse (< 3 months: refractory; < 6 months: resistance; 6 – 12 months: sensitive; 12 – 24 months: very sensitive) [37]. In addition to treatment-free interval, prediction of response includes a number of prior regimens, toxicity from prior therapy, previous use of growth factors and/or transfusions, performance status, volume of disease, number of disease site, ascites, and signs and symptoms of gastrointestinal dysfunction. At present, complete responses to treatment for recurrent disease are rare, particularly if the patient's time to relapse is short. Treatment-free intervals decrease after each relapse and retreatment, which may increase toxicities. The median survival after disease recurrence is in the range of 12 – 24 months [36]. As a general rule, the later the recurrence, the better the prognosis for survival duration. The aims of palliative treatment in relapsed ovarian cancer are, therefore, to control disease-related symptoms and minimise the side effects of treatment in order to prolong survival and delay time to progression. Maintenance or, preferably, improvement in quality of life becomes an important goal in these patients. A number of different strategies may be employed in the management of patients with relapsed ovarian cancer, including retreatment with platinum or salvage therapy with a variety of other agents, either alone or in combination regimens.

One treatment management option in relapsed patients is to reuse a platinum/taxane combination. However, response rates to such therapy are particularly low in patients with a short treatment-free interval. The correlation between platinum-free interval and response to second-line platinum

Table 1. Comparison of survival between adjuvant chemotherapy after initial debulking surgery and neoadjuvant chemotherapy followed by interval surgery.

	Comparison of survival	Comparison of debulking
Jacob (1991) [68]	Median survival	Optimal (%)
Adjuvant	18 months	39%
NAC therapy	16 months	77% (p = 0.02)
Onnis (1996) [70]	3- and 5-year	Optimal (%)
Adjuvant	survival	29%
NAC therapy	31 versus 21%	42%
	27 versus 19%	
Schwartz (1999) [71]	Median survival	
Adjuvant	2.18 years	
NAC therapy	1.07 years	
Vergote (1998) [72]	3-year survival	
Adjuvant	26%	
NAC therapy	42% (p = 0.001)	

NAC: Neoadjuvant chemotherapy.

combination therapy has been clearly demonstrated in number of studies [37-39]. The number of responders in the 6- to 12-months category is thought to be in the 25 – 30% range, slowly increasing to a rate of 60 – 70% at 2 years. Combinations of carboplatin and paclitaxel appear to have a higher response rate and may also blunt the platinum-free interval effect seen with single-agent platinum treatment [40]. This was also the result of the recently presented International Collaborative Ovarian Neoplasm 4 report [41]. The platinum-free interval has been used to classify relapsed patients for therapy. Essentially all agents appear to be more active in patients off therapy for > 6 months. Because all of these patients are currently incurable, the overall goal of therapy is to extend survival through a series of chronic treatments. The most beneficial sequence of treatments for particular patients has not been established.

A considerable number of nonplatinum agents have been investigated for the treatment of patients with relapsed ovarian cancer. Examples of efficacy with single-agent therapy with paclitaxel, topotecan (because topotecan is not approved in Japan, irinotecan is used), liposomal doxorubicin, etoposide and gemcitabine in recurrent ovarian cancer, as well as their known cumulative toxicities, have been shown [39-41,44-56].

Paclitaxel, a unique antimicrotubule agent, has been one of the most promising drugs to enter into clinical trials in the setting of cisplatin-refractory ovarian cancer. Responses have been reported in both heavily and minimally pretreated ovarian cancer patients (20 – 37%) [57]. However, myelotoxicity was found to be a major concern even with granulocyte colony-stimulating factor support. In order to minimise toxicity, paclitaxel can be given weekly instead of every 3 weeks [58,59]; this results in a higher dose intensity of the drug [58]. Two non-randomised trials [61,62] have suggested that the activity of paclitaxel in epithelial ovarian cancer is dose-dependent, and a randomised trial [58] has shown reduced toxicity with weekly scheduling without detriment to efficacy. It has been reported that single weekly paclitaxel has moderate activity in heavily pretreated ovarian cancer patients, and 80 mg/m^2 of paclitaxel was recommended as the Phase II dose for out-patients [63]. With 80 mg/m^2 of paclitaxel, the dose intensity may not be greater than once every three weeks. However, continuous low-dose paclitaxel has been reported to result in antiangiogenic effects and tumour dormancy [64,65]. Thus, the effects of single weekly paclitaxel in heavily pretreated patients with recurrent or persistent ovarian cancer were investigated. Thirty-seven patients were included in this intent-to-treat study. The overall clinical response rate was 45.9% (5 complete responses, 12 partial responses). The clinical response rate in patients with measurable tumour was 25.0% (2 complete responses, 1 partial response), whereas that in patients without measurable tumour and with assessable cancer antigen 125 (CA125) levels was 56.0% (3 complete responses, 11 partial responses). The criteria for response was based on declining CA125 levels as described by Rustin *et al.* [66]. Clinical response rates in patients with chemotherapy-free interval of > 6 months were around twice those found in patients with chemotherapy-free interval of < 6 months. The clinical response rate by number of prior regimens revealed that as number of prior regimens increases, the response rate decreases. Weekly paclitaxel has significant antitumour activity in heavily pretreated patients with recurrent or persistent ovarian carcinoma, and may be used as second- or third-line chemotherapy in such a setting [34]. Likewise, weekly administration of docetaxel has demonstrated comparable efficacy together with reduced myelosuppression in patients with solid tumours, including breast tumour, but not ovarian cancer [67].

4. Neoadjuvant chemotherapy

The clinical basis of aggressive cytoreductive surgery in the initial management of ovarian cancer is the significantly improved survival accrued to those patients in whom optimal cytoreductive surgery was accomplished [68,69]. The theoretical basis for primary cytoreductive surgery is supported by tumour cell growth kinetics observations that: an increase in cell-doubling time occurs as cancer becomes larger; resection of large tumour masses increases the number of residual cells that are in an active growth phase and are more sensitive to chemotherapy; and surgical cytoreduction results in an exponential reduction of tumour volume, thus leaving fewer cells to be eradicated [70]. These observations would suggest that neoadjuvant chemotherapy should, if anything, impair survival of women with advanced ovarian cancer. Some retrospective studies failed to demonstrate this as is shown in Table 1.

Although the prognosis for patients with advanced ovarian cancer has been improving over the last decades, long-term survival figures are still disappointingly low. More adequate therapeutic approaches need to be developed, especially for

patients whose tumours cannot be optimally debulked upfront. One such approach is the concept of chemical cytoreduction before debulking surgery in selected patients. Based on the available data, neoadjuvant chemotherapy in advanced ovarian cancer seems to allow for higher optimal debulking rates without compromising survival, and might be a valid alternative to upfront debulking surgery in patients with a high total metastatic load, stage IV disease, the presence of uncountable peritoneal metastases, or a poor performance status [71,72]. Some studies suggest that additional benefits may be reduced perioperative morbidity and increased quality of life. Hence, even if neoadjuvant chemotherapy followed by debulking surgery does not result in a better but similar overall survival compared with conventional treatment, it still may be a worthwhile approach based on considerations of morbidity, economic cost and quality of life. Some patients with primarily chemoresistant disease might also be spared the burden of an unnecessary laparotomy. All these issues have undoubtedly to be tested in a prospective randomised fashion. Until the results of such evaluations are available, neoadjuvant chemotherapy should not be considered as part of standard therapy in patients with advanced ovarian cancer, for whom the standard of care is still upfront maximal debulking surgery by an appropriately trained and experienced gynaecological oncologist.

5. Metronomic chemotherapy

Chemotherapeutic drugs, which have long been the mainstay of cancer treatment, cause DNA damage and disrupt DNA replication in proliferating cells. Drug regimens have been designated to kill as many tumour cells as possible by treating with MTDs of these cytotoxic agents. Side effects such as neurotoxicity and damage to proliferating cells in healthy tissues pose serious constraints on the use of chemotherapy. In an effort to balance toxicity with efficacy, a conventional dosing schedule calls for episodic application of a cytotoxicity drugs at or near the MTD, followed by periods of rest to allow normal tissues to recover. Many such chemotherapy regimens are initially efficacious, resulting in tumour regression or stabilisation and prolonged survival. In general, however, responses are short-lived, with relapses often marked by aggressive cancer that is resistant to the cytotoxic drug. Furthermore, the standard MTD regimen as a rule seriously impairs quality of life.

Although the collateral damage inflicted on the dividing bone marrow progenitors, gut mucosal or hair follicle cells by DNA damaging of microtubule inhibiting agents is certainly undesirable, the same cannot always be said of the damage inflicted on endothelial cells present in a tumour's growing neovasculature. A proportion of these cells are dividing at any given time, making them, at least in theory, sensitive to drugs that preferentially damage or destroy cycling cells [73]. Polverini's group first reported antiangiogenic effects mediated by conventional cytotoxic anticancer drugs as long ago as 15 years, and since then most common anticancer chemotherapeutic agents, belonging to all major classes, have been shown to be capable of inhibiting angiogenesis [74]. This prompted Sledge and colleagues [64] recently to suggest the notion of 'redefining' chemotherapeutic drugs as antiangiogenics. It is intriguing and perhaps reassuring to note that there are many clinical precedents for the observations of Browder *et al.*, as summarised recently by Kamen *et al.* [75], and by Gately and Kerbel [76]. For example, significant proportions of breast and ovarian cancer patients (≤ 62.5%) who had stopped responding to MTDs of a taxane given once every 3 weeks, were subsequently found to respond to the same drug once it was switched to a weekly schedule at about a third of the MTD [58,77-79]. Such weekly schedules using lower drug doses were instituted to minimise the toxicities associated with once-every-3-weeks MTD taxane protocols. It is not yet known whether the rsesponses observed in these 'resistant' patients have an antiangiogenic basis, or whether such increased response rates will translate into a significant prolongation of survival, as they do in mice [80,81].

Introduction of paclitaxel into the armamentarium of drugs to treat platinum-resistant ovarian cancer has been one of the more significant advances in the treatment of ovarian cancer in the last decade. Paclitaxel has a unique mechanism of action, is cell-cycle-specific, and acts by promoting the stability of the microtubule assembly during mitosis. *In vitro* data suggest that the duration of exposure plays a crucial role in the cytotoxicity efficacy of paclitaxel [82,83]. Resistance to paclitaxel-mediated P-glycoprotein [84] has been shown to be significantly reduced by increasing the duration of exposure to paclitaxel from 3 to 96 h in P-glycoprotein-expressing paclitaxel-resistant breast cancer cell lines [85]. Weekly administration of paclitaxel has the potential to have an effect similar to that of continuous infusion while taking advantage of the minimal haematological toxicity associated with shorter infusions [34]. Neutropenia was the most frequent haematological adverse event observed in patients receiving once-weekly intravenous paclitaxel monotherapy. Severe neutropenia was dose-related, occurring only in 3 – 15% of patients receiving 80 mg/m^2 monotherapy [86,87]. An absolute neutropenia count of 1000 has been shown to be sufficient for dosing weekly paclitaxel on any given scheduled day of treatment. In this study, severe neutropenia and leukopenia of grade 4 were observed in 2 (5.4%) and 1 (2.7%) of 37 patients, respectively. Other haematological adverse events such as grade 4 anaemia and/or grade 4 thrombocytopenia were not observed. Neuropathy is experienced by most patients receiving once-weekly intravenous paclitaxel monotherapy and is usually mild or moderate [86,87]. Treatment with single weekly 80 mg/m^2 paclitaxel brought about an overall response rate of 45.9%, which is similar to that of a recent report [88]. It is noteworthy that 5 complete responses among 37 patients with one or more therapeutic regimens were achieved.

The choice of second-line drug in this present setting is dependent on toxicity and quality of life considerations, in

Table 2. HER-2/neu and EGFR overexpression rate according to histological type.

Histology	HER-2/neu overexpression	EGFR overexpression
Serous	8/60 (13%)	24/60 (40%)
Endometrioid	0/15 (0%)	4/15 (27%)
Mucinous	2/11 (18%)	2/11 (18%)
Clear	6/26 (23%)	11/26 (42%)
Total	16/112 (15%)	41/112 (36%)

EGFR: Epidermal growth factor receptor; HER-2: Human epidermal growth factor receptor 2.

addition to efficacy. Weekly administration of paclitaxel by 1-h infusion has been reported to have less toxicity than other schedules and primary effect in patients with pretreated gynaecologic cancers [58,60,89,90]. In addition, a randomised Cancer and Leukemia Group B trial comparing the weekly schedules to paclitaxel given once every 3 weeks for advanced breast cancer is nearing completion. 'Metronomic' dosing or antiangiogenic scheduling of cancer chemotherapeutics has been increasingly recognised to be a potential application of paclitaxel in cancer therapy [91-93].

6. Molecular-targeted chemotherapy

Traditional cytotoxic agents cannot distinguish malignant from nonmalignant cells. As a result, use of these agents at clinically effective doses is often accompanied by severe toxicity. This lack of specificity has stimulated the development of a new breed of agents that primarily target growth and signalling processes in malignant cells and, thus, tend to be less toxic to normal cells than conventional cytotoxic therapies [94]. These specially engineered compounds largely target cell-membrane receptors that control the intracellular signal transduction pathways regulating cell proliferation and apoptosis, angiogenesis, cellular adhesion and cell motility.

6.1 Epidermal growth factor receptor inhibitors

The epidermal growth factor receptor (EGFR) is highly expressed in a variety of solid tumours, including ovarian cancer. Activation of the EGFR signalling pathways has been linked with increased cell proliferation, angiogenesis, metastasis and decreased apoptosis [95]. Preclinical studies have shown that blocking this pathway inhibits these processes both *in vitro* and *in vivo* and increases apoptosis of malignant cells, while having minimal effects on normal cell function. The authors' clinical studies revealed that overexpression of EGFR was observed in 36% of ovarian cancer and seemed to be greater in serous cystadenocarcinoma and clear cell carcinoma than in endometrioid adenocarcinoma and mucinous cystadenocarcinoma, although not significant (Table 2).

The anti-EGFR therapies currently undergoing clinical development are the monoclonal antibodies trastuzumab (Herceptin®, Genentech, Inc.) and cetuximab and small-molecule EGFR tyrosine kinase inhibitors gefitinib and erotinib (OSI-774/Tarceva™, OSI Pharmaceuticals, Inc.). Proliferation of ovarian epithelial cancer cells expressing HER-2/neu is blocked by trastuzumab *in vitro* [96], and the results of clinical testing at Ohio State University in ovarian cancer patients were shown to be inactive because of a small percentage of HER-2/neu-overexpressing tumours. In an immunohistochemical study, rate of HER-2/neu overexpression in ovarian cancer was 15%, and it is noteworthy that overexpression of HER-2/neu in endometrioid carcinoma was not observed, whereas clear cell carcinoma showed a higher staining rate (Table 2). A Phase I study of its safety in patients with a variety of tumours, including ovarian cancer, established that the drug was well-tolerated at doses of ≤ 600 mg/day and that treatment inhibited the EGFR signalling pathway [97].

Objective antitumour responses and evidence of disease stabilisation were documented in 34 patients with advanced platinum- and/or paclitaxel-resistant ovarian cancer who had been treated with erotinib [98].

6.2 Signal transduction inhibitors

Aberrant signal transduction has been implicated in malignant transformation, growth and progression. This has led to the proposal to use inhibitors of signal transduction pathways to treat cancer. Chronic myelogenous leukaemia (CML), for example, is characterised by a translocation between chromosomes 9 and 22. The fusion of the *Abl* gene on chromosome 9 with the *Bcr* gene on chromosome 22 forms a *Bcr-Abl* fusion gene that expresses tyrosine kinase, which is thought to be leukaemogenic. Imatinib mesylate (STI-571/Gleevec®, Novartis Pharmaceuticals Corporation) is a potent inhibitor of *Bcr-Abl* tyrosine kinase and selectively kills *Bcr-Abl*-expressing tumour cells. Recent studies have shown that several tumours express c-KIT: a growth factor receptor with tyrosine kinase activity; moreover, clinical results have shown the efficacy of the tyrosine kinase inhibitor, imatinib mesylate, in c-KIT-positive tumours. Intense c-KIT immunostaining was observed in 51.7% of cases. c-KIT expression was statistically correlated with progression of disease after first-line chemotherapy. c-KIT is also expressed in ovarian carcinoma and it is statistically correlated with chemotherapy resistance. Clinical trials confirming the utility of the tyrosine kinase inhibitor, imatinib mesylate, in advanced ovarian cancer patients with c-KIT overexpression who have shown no clinical response to conventional chemotherapy are warranted [99]. Clinical trials of imatinib mesylate in ovarian cancer are being conducted by the Gynecologic Oncology Group (GOG), National Cancer Institute and the Southwest Oncology Group. The PI3K/AKT pathway stimulates cell proliferation, inhibits apoptosis and increases drug resistance. The upregulation of the P110-α catalytic subunit of PI3K is often found in human ovarian cancer [100]. Kudoh *et al.* (pers. commun.) observed marked sensitising effect of PI3K inhibitor LY-294002 (Calbiochem) on antitumour effect of paclitaxel in a

paclitaxel-resistant human ovarian cancer cell line. The synergistic augmentation of the cytotoxocity by PI3K inhibitor LY-294002 occurs specifically with antimicrotubule agents, at least partially through an increase in caspase 3-dependent apoptosis, so that inhibitors of the PI3K/AKT pathway in combination with antimicrotubule agents may induce cell death effectively and be a potent modality to treat patients with malignant tumours [101]. PI3K inhibitor is a promising therapy strategy in drug-resistant ovarian cancer [102].

6.3 Antiangiogenesis therapy

Angiogenesis, the formation of new blood vessels, is essential to the growth and proliferation of solid tumours. Presumably, anything that interferes with angiogenesis will cause the tumour to 'starve' and eventually kill it, a concept originally proposed by Folkman [103]. Tumour angiogenesis may be regulated by angiogenic factors such as VEGF [104] and IL-8 [105]. Of the known proangiogenic factors, VEGF is one of the most potent and specific, and it has been identified as a crucial regulator of both normal and pathological angiogenesis. Overexpression of VEGF has been demonstrated in most human cancers, including ovarian tumours. Bevacizumab is a recombinant anti-VEGF monoclonal antibody that recognises all biologically active isoforms of VEGF and blocks their binding to VEGF receptors, thus inhibiting angiogenesis [104]. A Phase II clinical trial, designed and implemented by the GOG protocol 170D, is currently underway to access the safety and efficacy of bevacizumab in patients with recurrent or persistent ovarian cancer. Also being investigated as a potential antiangiogenesis agent in ovarian cancer is thalidomide, which is showing some benefit in women refractory to conventional chemotherapy [106], and RPI-4610 (Angiozyme, Sirna Therapeutics, Inc.), a proprietary ribozyme that can downregulate VEGF receptor function by specifically cleaving the mRNA for a primary VEGF receptor: FLT-1. Clinical trials are currently in progress to establish the therapeutic efficacy and safety of RPI-4610 in patients with advanced malignancies. Extensive preclinical studies have demonstrated no significant toxicities [107]. Another antiangiogenic molecule under development is the PKC-β inhibitor LY-317615. This small, orally available molecule has demonstrated the ability to inhibit growth-factor-driven proliferation of tumour neovascularisation and is currently undergoing Phase I testing in several tumour types [108]. Recently, it has been reported that bisphosphonates (pamidronate) induce significant and lasting modifications of angiogenic cytokine patterns [109]. Experimental trials should be addressed to assess the real clinical impact in anticancer therapy of antiangiogenic properties of bisphosphonates.

The inducible enzyme cyclooxygenase-2 (COX-2) is an important mediator of angiogenesis and tumour growth. Selective COX-2 inhibitor drugs, commonly prescribed for pain management, are now being evaluated for their antitumour and antiangiogenic activities. These drugs include celecoxib (Celebrex®, Pfizer, Inc.), rofecoxib (Vioxx®, Merck & Co, Inc.) and valdecoxib (Bextra®, Pfizer, Inc.). Oral celecoxib (30 mg/kg/day) inhibited angiogenesis by 79% in a rat model of basic fibroblast growth factor (bFGF)-induced cornel angiogenesis, and reduced corneal levels of prostaglandin E2 and thromboxane 2 by 79 and 68%, respectively [110]. Celecoxib can also inhibit angiogenesis via COX-2-independent mechanisms. Impaired VEGF gene expression and decreased angiogenesis result from celecoxib-induced interference with DNA binding of the Sp1 transcription factor [111]. Celecoxib has also been reported to increase serum levels of the endogenous angiogenesis inhibitor endostatin, while decreasing the release of VEGF by platelets [112], thus altering the balance of angiogenesis regulation in favour of inhibition. A Phase II study of lung cancer patients receiving celecoxib 400 mg b.i.d. p.o. concurrently with paclitaxel/carboplatin plus radiation therapy found that serum/plasma levels of VEGF declined at 2, 5 and 7 months following treatment [113]. Rofecoxib also has been shown to inhibit angiogenesis in a number of *in vivo* systems. Administration of rofecoxib blocks the production of bFGF and reduces wound healing angiogenesis in experimental gastric ulcers [114]. In a model of retinopathy, rofecoxib inhibited neovascularisation in COX-2-expressing retinal vessels [115]. Based on supportive preclinical data, a large-scale clinical trial is underway in Europe studying rofecoxib as an adjuvant antiangiogenic treatment in 3500 patients with previously resected colorectal cancer. Although no clinical trials in ovarian cancer have been carried out, trials in such an adjuvant setting are awaited.

7. Conclusion

The management of ovarian cancer begins with appropriate surgical staging. Following surgical staging and removal of the reproductive organs, adjuvant chemotherapy has been performed. The standard regimen over the past several years has been a combination of carboplatin (area under the curve: 5 – 7.5) plus paclitaxel (175 mg/m^2, infused over 3 h). Studies carried out by GOG, as well as several European trials, have demonstrated optimum response rates with this combination, and it has come to be accepted as the 'gold standard' for treating ovarian cancer. Although this regimen has resulted in prolongation of survival times, only modest improvement of overall survival has been observed with this treatment strategy.

Recurrent ovarian cancer patients with platinum-refractory disease can still respond to platinum retreatment following treatment with continuous low-dose paclitaxel. In patients with platinum-resistant disease the use of intervening therapy to extend the platinum-free interval may be a useful strategy, providing a similar immediate response rate and an improved response to platinum later.

At present, solid evidence demonstrating the superiority of neoadjuvant chemotherapy followed by postdebulking chemotherapy over conventional postdebulking chemotherapy alone is lacking, but further study is needed. Elderly and medically

compromised patients with massive ascites are excellent candidates for neoadjuvant chemotherapy, as it avoids postoperative fluid shifts, which can stress the cardiovascular integrity of these patients.

Some patients who are receiving long-term maintenance or even palliative chemotherapy continue to have stable disease beyond the time that the tumour cells would have been expected to develop drug resistance. A closer approximation to antiangiogenic scheduling may explain the improved outcome of empiric treatment of 'slower growing' human cancer using continuous infusion 5-fluorouracil in breast cancer and colorectal cancer [116-118], weekly paclitaxel in recurrent ovarian cancer and pretreated solid tumours [119,120], and daily oral etoposide in non-small cell lung cancer and in supratentorial malignant glioma in children [121-123]. If this hypothesis proves generalisable, it may suggest which agents and on which schedules chemotherapy may be best combined with more specific angiogenesis inhibitors for improved antiangiogenic and anticancer efficacy.

Molecular-targeted therapy could be considered, using novel agents capable of homing in on a single molecular target that is overexpressed in cancer cells, but lacking in normal cells. These gene- and target-based therapies are able to become new treatment strategies with less toxicity than conventional treatment modalities. The application of these new treatment strategies to ovarian cancer is still in its infancy. Recently, it has been reported that in a stringent preclinical model, standard chemotherapy followed by a novel maintenance regimen resulted in disruption of pericyte support by plasmid-derived growth factor receptor and subsequent metronomic chemotherapy and/or VEGF receptor inhibitors target consequently sensitised endothelial cells, collectively destabilising pre-existing tumour vasculature and inhibiting ongoing angiogenesis [124]. This exciting translational work requires many disciplines and organisations to work together internationally to accelerate patient benefit.

8. Expert opinion

Poor prognosis of ovarian cancer compared with uterine cervical cancer and endometrial cancer is due to incapability of early diagnosis. Ovarian cancer presents at a late clinical stage in > 75% of patients, and is associated with a 5-year survival of 35% in this population. By contrast, the 5-year survival for patients with Stage I ovarian cancer is > 90%, and most patients are cured of their disease by surgery alone. Therefore, increasing the number of women diagnosed with Stage I disease should have a direct effect on the mortality and economics of this cancer without the need to change surgical or chemotherapeutic approaches. A global view of the proteome would enhance the possibility of identifying protein signatures for ovarian cancer. Surface-enhanced laser desorption and ionisation with time of flight detection (SELDI-TOF) spectral analysis was linked with a high-order analytical approach using samples from women with a known diagnosis to define an optimum discriminatory proteomic pattern. This pattern was used to predict the identity of masked samples from unaffected women, women with early and late-stage ovarian cancer, and women with benign disorders. Following proper validation, serum proteomic pattern analysis might be ultimately applied in medical screening clinics, as a supplement to the diagnostic workup and evaluation. A negative value, if the sensitivity remains at 100% on further trials, could be used for reassurance, whereas a positive value may be sufficient to warrant further evaluation. An important future goal is confirmation of sensitivity and specificity for the prospective detection of Stage I ovarian cancer in trials of high- and low-risk women, respectively. It will be important to design the trial to evaluate the efficacy of the approach as a standalone approach or one to be combined with current screening options. Such trials should benefit patients, particularly ovarian cancer patients.

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Clinical significance of heparin-binding epidermal growth factor-like growth factor in peritoneal fluid of ovarian cancer

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Epidermal growth factor receptor (EGFR) has been implicated in tumour growth and extension of ovarian cancer. Peritoneal fluid in ovarian cancer patients contains various growth factors that can promote tumour growth and extension. In order to investigate the clinical significance of EGFR ligands as activating factors of ovarian cancer, we examined the cell proliferation-promoting activity and the level of EGFR ligands in peritoneal fluid obtained from 99 patients. Proliferation-promoting activity in peritoneal fluid from 63 ovarian cancer patients (OVCA) was much higher than peritoneal fluid from 18 ovarian cyst patients (OVC) and 18 normal ovary patients (NO), and the activity was suppressed only by antibodies against EGFR or heparin-binding epidermal growth factor (HB-EGF). A large difference was observed in the level of EGFR ligands between HB-EGF and TGF-α or amphiregulin. The concentration of HB-EGF in OVCA significantly increased compared to that in OVC or NO ($P<0.01$). No significant difference in the concentration of TGF-α and amphiregulin was found between the OVCA and NO or OVC groups. In peritoneal fluid, HB-EGF is sufficiently elevated to activate cancer cells even at an early stage of OVCA. These results suggested that HB-EGF in peritoneal fluid might play a key role in cell survival and in the proliferation of OVCA.
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Ovarian cancer (OVCA) is the most frequent cause of cancer death among all gynaecologic cancers, and in the last 30 years current therapies have not improved cure rates (Penson *et al*, 1998). The high mortality is caused predominantly by the occult progression of the tumour into the peritoneal cavity with an initial diagnosis usually being made at an advanced stage. Tumour growth is characterised by local extension into the peritoneal cavity following the circulatory pathway of the peritoneal fluid produced by peritoneal epithelium and cancer cells. Accumulated evidence from many studies reveals that ascites from OVCA patients is a rich source of growth factor activity for OVCA cells, termed ovarian cancer activating factors (OCAFs) (Mills *et al*, 1998). The dissemination of cancer cells activated by OCAFs result in an exaggerated increase in peritoneal fluid, which in turn leads to tumour extension of OVCA.

To identify OCAFs of OVCA, various peptide growth factors and cytokines have been detected in the malignant effusions of OVCA patients (Westermann *et al*, 1997). However, the growth-promoting properties of malignant effusions *in vivo* and *in vitro* have been shown to be independent of these peptide growth factors (Westermann *et al*, 1997). Recent biochemical analysis has revealed that one possible OCAF candidate is lysophophatidic acid (LPA) (Xu *et al*, 1995; Westermann *et al*, 1998; Xiao *et al*, 2001). Lysophophatidic acid is a simple phospholipid with numerous cellular effects including growth promotion, cell cycle progression and cytoskeletal organisation (Mills and Moolenaar, 2003). However, LPA may not be the sole mediator present in ascites because the proliferation of cancer cells was lower than that induced by ascites from OVCA patients, even at optimal LPA concentrations (Xu *et al*, 1995).

Impairment of the epidermal growth factor (EGF) system has been implicated in the pathogenesis of different types of carcinomas (Salomon *et al*, 1995; Normanno *et al*, 2003). As described in the literature (Salomon *et al*, 1995; Normanno *et al*, 2003), EGF receptor (EGFR) overexpression occurs in 35–70% of all primary OVCAs and the overexpression of ErbB2 is correlated to clinical outcome. Whereas the frequency of ErbB2 overexpression is low, the frequencies of ErbB3 and ErbB4 expressions are high in OVCA. Univariate and multivariate statistical analyses have confirmed that EGFR overexpression is significantly associated with a high risk of progression in OVCA patients (Scambia *et al*, 1992). Seven ligands have been described for EGFR: EGF, transforming growth factor-α (TGF-α). heparin-binding-EGF like growth factor (HB-EGF), amphiregulin (AR), betacellulin, epiregulin, and epigen (Fischer *et al*, 2003). All are synthesised as membrane-spanning precursor molecules that have to be proteolytically processed to become fully active (Fischer *et al*, 2003). A relatively high frequency of TGF-α and AR has been described in ovarian carcinomas, although staining in tumours varied from weak to

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strong (Morishige *et al*, 1991; D'Antonio *et al*, 2002). Ovarian cancer cells are sensitive to diphtheria toxin, indicating the expression of proHB-EGF (Morimoto *et al*, 1991). We reported that HB-EGF is involved in EGFR signal transactivation induced by LPA in OVCA cell lines, and that the soluble form of HB-EGF is attributable to tumour growth on xenografted mice using OVCA cell lines (Miyamoto *et al*, 2004). On the basis of these results, it is suggested that EGFR plays a pivotal role in the acceleration and progression of ovarian caner through EGFR ligands including TGF-α, HB-EGF, and AR.

The concentration of EGFR ligands was examined to determine their role as tumour-promoting factors in the peritoneal fluid of OVCA patients. Peritoneal fluid of OVCA patients was also examined for proliferation-promoting and cell survival activities in OVCA cells in the absence or presence of specific inhibitory antibodies against EGFR and EGFR ligands.

MATERIALS AND METHODS

Patients and peritoneal fluids

All of the 99 patients in this study underwent surgery between 1994 and August 2003 at the Department of Obstetrics and Gynecology, Kyushu University Hospital. Peritoneal fluids were obtained from 99 women, who gave signed informed consent (Table 1). In all, 18 cases with normal ovaries had surgical treatment due to uterine myomas or benign gynaecologic disorders. In six cases with marked ascites and multiple disseminating sites in the peritoneum, peritoneal fluids were obtained twice, once before chemotherapy and once after three courses of chemotherapy, under a presurgical state. Peritoneal fluid supernatants were collected immediately after centrifugation (3000 g × 15 min), and stored at −80 °C until use.

Reagents

Recombinant human HB-EGF and synthetic LPA were purchased from R&D Systems Inc. (Minneapolis, MN, USA) and from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), respectively. [^{3}H]thymidine (6.7 Ci mmol^{-1}) was obtained from New England Nuclear (Lachine, Quebec, Canada). Mouse anti-human EGF receptor neutralising antibody and goat anti-human HB-EGF neutralising antibody was obtained from Upstate Inc. (Lake Placid, NY, USA) and R&D Systems Inc. (Minneapolis, MN, USA), respectively. Goat anti-human TGF-α neutralising antibody, mouse anti-human AR neutralising antibody, mouse anti-human EGF neutralising antibody, goat anti-human epiregulin neutralising antibody and goat anti-human betacellulin neutralising antibody were also purchased from GT (Minneapolis, MN, USA). The manufacturer's instructions detail that at least 5 ng ml^{-1} of EGFR and EGFR ligands (EGF, TGF-α, HB-EGF, AR, betacellulin and epiregulin) can be neutralized in the use of 10 μg ml^{-1} of these antibodies, respectively.

Table 1 Clinical data for patients

Variable	No. of patients	Age (years) (mean ± s.d.)	Histological types: Serous	Histological types: Others
Normal ovary	18	55.5 ± 17.8		
Ovarian cyst	18	54.6 ± 19.7	10	8
Ovarian cancer, stage Ia	10	57.2 ± 14.3	7	3
Ovarian cancer, stage Ic–II	13	55.8 ± 10.6	8	5
Ovarian cancer, stage III–IV	30	59.3 ± 16.2	22	8
Ovarian cancer, recurrence	10	58.2 ± 11.2	7	3

Polyclonal rabbit anti-EGFR and anti-ErbB-4 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Upstate Biotechnology Inc. (Lake Placid, NY, USA), respectively. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Zymed (San Francisco, CA, USA).

Immunoblot

Cells were rinsed in phosphate-buffered saline (PBS) and then lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris (pH 8.0), 0.2 unist ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 2 mM phenylmethylsulphonyl fluoride). In all, 50 μg of extracts wsa then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis (Miyamoto *et al*, 2004).

Cell proliferation-promoting assay mediated by factors in peritoneal fluid

SKOV3 cells, derived from OVCA, were maintained in RPMI-1640 (Nacalai Tesque Co. Ltd., Kyoto, Japan) supplemented with 10% (v v^{-1}) fetal bovine serum (FBS). To remove extracellular matrix components, cells were detached with trypsin-EDTA, then allowed to recover for 30 min in RPMI-1640 with 10% FBS. After rinsing with serum-free medium, cells were incubated with serum-free medium at 37 °C for 30 min. Cells (1×10^4) were seeded on poly-lysine-coated dishes, and samples incubated with serum-free RPMI-1640 at 37 °C for 1 h to assure the complete adherence of cells to the poly-lysine-coated dishes. To assess cell proliferative activity, cells were incubated in 200 μl of RPMI-1640 plus 90% of each human peritoneal fluid at 37 °C for 24 h; then WST-1 assay (Dojin Laboratory, Kumamoto, Japan) was performed according to the manufacturer's instructions. Further, to reconfirm the potential of the DNA polymerisation induced by peritoneal fluids, [^{3}H]thymidine incorporation was examined in SKOV 3 cells, using 10% human peritoneal fluid. The cells were treated in the same manner as the WST-1 assay, and the medium was then replaced with RPMI-1640 plus 10% of each human peritoneal fluid for an additional 24 h in the absence or presence of inhibitory antibodies against EGFR, HB-EGF, TGF-α, amphiregulin, epiregulin, betacellulin, and EGF, or with RPMI-1640 plus 10% peritoneal fluid from patients with a normal ovary (NO) in the absence or presence of various concentrations of LPA or HB-EGF. Peritoneal fluids from 30 patients with OVCA (five cases at stage Ia, five at Ic-II, 15 at III–IV, and five of recurrence) were available for the experiment using inhibitory antibodies. [^{3}H]thymidine (1 μCi well^{-1}) was then added to the cell culture. After 4 h of labelling with [^{3}H]thymidine, cells were washed with PBS, lysed with NaOH and treated with TCA. After adding scintillation fluid, [^{3}H]thymidine uptake was measured in a β-scintillation counter. Each experiment was conducted in triplicate. The mean value was considered as the representative value for each experiment. The WST-1 assay and the [^{3}H]thymidine incorporation were also examined in other OVCA cell lines including RMG-1 and OVMG1 cells in 10 cases with a NO and in 20 cases with OVCA.

Inhibition of cell apoptotic assay mediated by factors in peritoneal fluid

The OVCA cell lines (1×10^5) of SKOV3, RMG-1, and OVMG1 were treated in the same manner as in the WST-1 assay. Thereafter, this medium was replaced with the RPMI-1640 plus 10% of each human peritoneal fluid for an additional 24 h in the absence or presence of inhibitory antibodies against HB-EGF. Cells were harvested, pooled, and then fixed with 4% paraformaldehyde and 70% ethanol. After further washing in PBS, cells were incubated with TdT reaction reagent for 1 h at 37 °C, according to the manufacturer's recommended protocol (MEBSTAIN Apoptosis Kit

Direct, MBL, Co., Ltd, Japan). TUNEL-positive cells were quantified as apoptotic cells by flow cytometric analysis (Becton Dickinson, FACScan, 01-20126-xx, USA).

Binding assay for HB-EGF

The binding of ^{125}I-diphtheria toxin (DT) to HB-EGF was measured as described previously (Iwamoto *et al*, 1994). Briefly, 1 ml of peritoneal fluid was incubated with heparin-sepharose CL-6B for 5 h at 4°C. The gel was washed three times with PBS and incubated with ^{125}I-DT in the presence or absence of excess unlabelled DT for 12 h at 4°C. It was then washed three times with PBS and three times with high-salt PBS. The radioactivity bound to the gel was counted with a gamma counter, and the specific binding of ^{125}I-DT to the HB-EGF molecule was calculated by subtracting the radioactivity of the sample in the presence of unlabelled DT from that of the sample in the absence of unlabelled DT. The amount of HB-EGF was estimated by the standard curve obtained using recombinant human HB-EGF. All experiments were conducted in triplicate and the mean HB-EGF value was regarded as the representative value of HB-EGF in each case.

Immunoassay for human TGF-α and AR

Concentrations of TGF-α and AR in peritoneal fluid were determined with a commercially available ELISA (Quantikine Kit, R&D Systems Inc. and ELISA Development Kit, GT, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. Samples were analysed in triplicate. Levels of TGF-α and AR were calculated from the linear areas of the standard curves obtained using Multiskan MS, version 8.0 (Labsystems, Helsinki, Finland), respectively. The mean value was used as the representative value. The lower limits for detection of TGF-α and AR were 5 and 10 pg ml^{-1}, respectively. When the amount was less than the detection limit, the TGF-α or AR value was recorded as 5 or 10 pg ml^{-1}, respectively.

Statistical analysis

The statistical significance was assessed using the Mann–Whitney test and a *P*-value less than 0.05 was considered statistically significant.

RESULTS

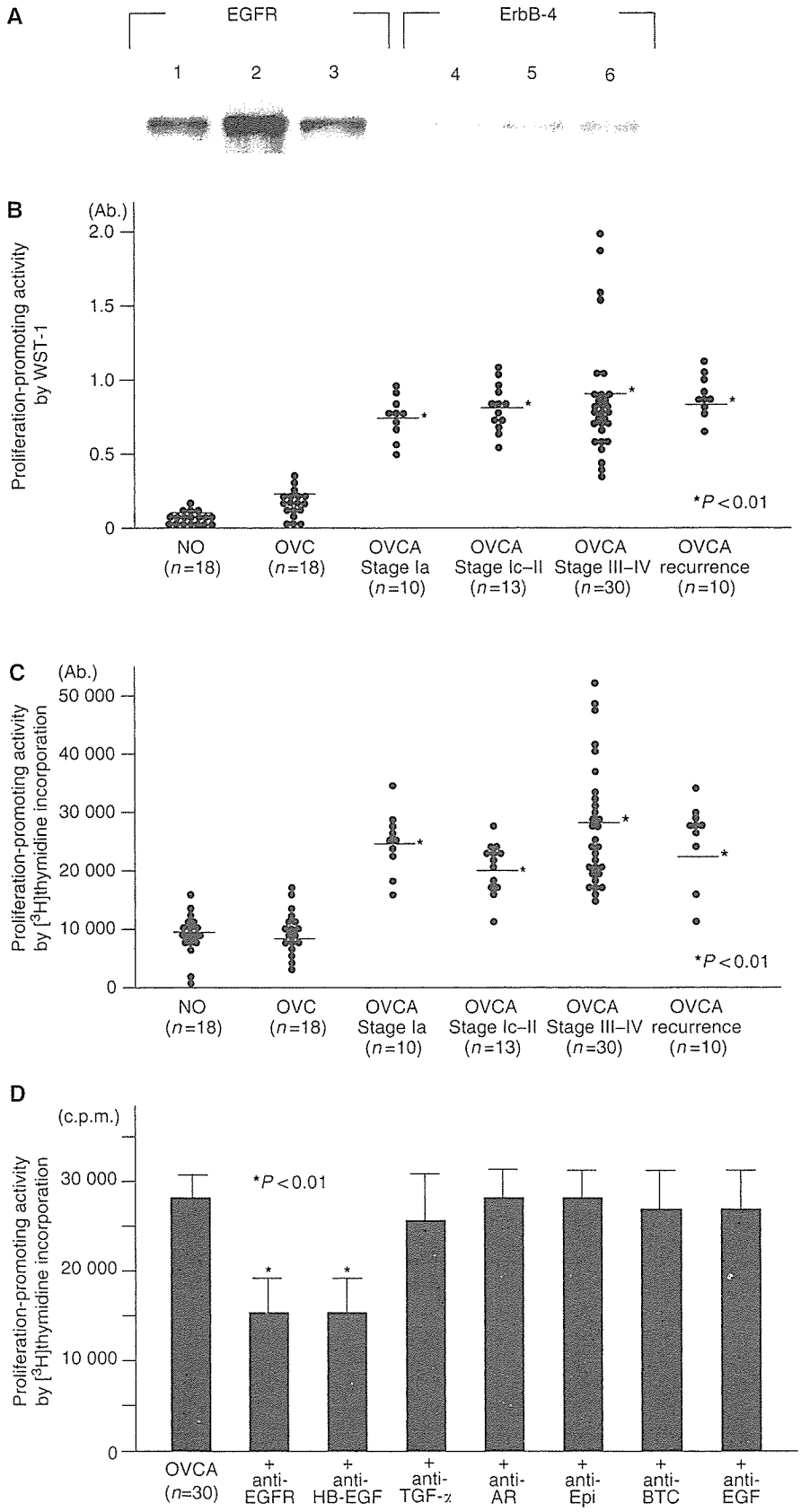
To investigate the expression of EGFR and ErbB-4 in the OVCA cell lines of SKOV3, RMG-1, and OVMG1, each protein expression was examined using immunoblotting analysis. The expression of EGFR in RMG-1 cells increased remarkably compared to those in SKOV3 or OVMG1 cells. There was little difference in the expression of ErbB4 among these three lines of OVCA cells (Figure 1A).

To assess the cell proliferative property mediated by factors in peritoneal fluid, cell proliferation of SKOV3 cells was examined using the WST-1 assay after incubation with peritoneal fluid. WST-1 assay demonstrated that the value in patients with OVCA significantly increased compared to that in patients with a NO or ovarian cyst (OVC) (Figure 1B and Table 2). To address whether peritoneal fluid from OVCA patients actually stimulates cancer cell proliferation, the proliferation of SKOV3 cells was examined by [^{3}H]thymidine incorporation into DNA. In the absence of the patient's peritoneal fluid, SKOV3 cells showed [^{3}H]thymidine incorporation at a minimum value (5800 ± 1200 cpm). Addition of peritoneal fluid resulted in increased DNA synthesis in all cases, but peritoneal fluid from OVCA patients enhanced DNA synthesis much highly than that from NO and OVC (each $P<0.01$) (Figure 1C and Table 2). In the WST-1 assay, the absorbances of the 10 cases with a NO and the 20 cases with OVCA were 0.31 ± 0.12 and 0.62 ± 0.23 (mean ± standard deviation) (RMG-1 cells), and 0.30 ± 0.13 and 0.69 ± 0.23 (OVMG1 cells), respectively. In RMG-1 and OVMG1 cells, the proliferative activity in peritoneal fluid of OVCA patients was significantly enhanced, compared with that in NO (both $P<0.01$). In [^{3}H]thymidine incorporation, the values in the 10 cases with a NO and the 20 cases with OVCA were 5823 ± 1066 c.p.m. (count per minute) and 9241 ± 1553 c.p.m (mean ± standard deviation) (RMG-1 cells), and 6903 ± 1134 c.p.m. and 12572 ± 1951 c.p.m. (OVMG1 cells), respectively. In RMG-1 and OVMG1 cells, the DNA polymerisation property mediated by peritoneal fluid in OVCA was also significantly elevated, compared with that in NO (both $P<0.01$). These results indicate that peritoneal fluid in patients with OVCA possesses a significant cell proliferative property.

To examine which EGFR ligands contribute to SKOV3 cell proliferation in a patient's peritoneal fluid, the effects of peritoneal fluid on SKOV3 cell DNA synthesis were measured in the absence or presence of an inhibitory antibody against EGFR or each EGFR ligand. [^{3}H]thymidine incorporation was significantly reduced in the presence of anti-EGFR or anti-HB-EGF neutralising antibody, while neutralising antibodies against TGF-α, AR, epiregulin, betacellulin, and EGF had no effect on [^{3}H]thymidine incorporation in SKOV3 cells (Figure 1D). The contribution of epigen was not determined, as no antibodies against epigen are available. In the presence of inhibitory antibodies against HB-EGF or EGFR, the values of [^{3}H]thymidine incorporation were 7301 ± 1093 and 7308 ± 1120 c.p.m (RMG-1 cells), and 9513 ± 1510 and 9160 ± 1296 c.p.m. (OVMG1 cells), respectively. [^{3}H]thymidine incorporation was also significantly reduced in the presence of anti-EGFR or anti-HB-EGF neutralising antibodies using RMG-1 or OVMG1 cells.

To evaluate the role of HB-EGF in cell survival in peritoneal fluid, apoptotic cells were analysed using the FACScan after staining cells by TUNEL methods in 10% human peritoneal fluid with an absence or presence of an inhibitory antibody against HB-EGF. Significant apoptotic cells in RMG-1 and OVMG1 cells were not observed even under serum-free conditions. In SKOV3 cells, 25.1 ± 1.4% (mean ± standard deviation) were detected as TUNEL-positive under serum-free conditions (Figure 2C). In a patient with a NO, there was no difference in the percentage of apoptotic cells between an absence or presence of peritoneal fluid, or between an absence or presence of the inhibitory antibody plus peritoneal fluid (Figure 2A). In a patient with OVCA, the percentage of apoptotic cells incubated with peritoneal fluid markedly decreased, compared to that incubated without peritoneal fluid (Figure 2B). As well, peritoneal fluid plus the inhibitory antibody against HB-EGF blocked any decrease of apoptotic cells (Figure 2B and C). In the 10 cases with a NO or the 20 cases with OVCA, the percentages of apoptotic cells were 24.6 ± 4.6 or 3.3 ± 2.2%, respectively, after incubation with peritoneal fluid (Figure 2C). In peritoneal fluid from OVCA patients in the presence of the inhibitory antibody against HB-EGF, the percentage of apoptotic cells significantly increased at 18.2 ± 5.2%, compared with that in the absence of the inhibitory antibody ($P<0.01$) (Figure 2C). These results suggest that HB-EGF in the peritoneal fluid of OVCA patients may contribute to cell survival in OVCA cells.

The present study suggests that HB-EGF levels may increase in peritoneal fluid from OVCA patients, and that HB-EGF is one of the factors in peritoneal fluid from OVCA patients that promotes tumour growth. Thus, HB-EGF, in addition to LPA, should be included as a member of OCAFs. To compare HB-EGF with LPA for proliferation-promoting activity, SKOV3 cells were cultured either with HB-EGF or LPA in a culture medium containing peritoneal fluid of NO patients. Although the concentration of LPA in the peritoneal fluid of OVCA patients



is reportedly from 10 to 20 μM (Xu *et al*, 1995; Westermann *et al*, 1998; Xiao *et al*, 2001), LPA (0–50 μM) did not show any growth-promoting effect in the present cell system (Figure 3A). In contrast, HB-EGF enhanced SKOV3 cell proliferation in a dose-dependent manner (Figure 3B), even at concentrations of 1–10 ng ml^{-1}, which are comparable to the levels of HB-EGF in peritoneal fluid of OVCA patients. These results indicate that HB-EGF induced significant cell proliferation, even in the presence of a concentration of 1 ng ml^{-1}.

To gain an insight into the role of EGFR ligands in peritoneal fluid of OVCA patients, the concentrations of HB-EGF, TGF-α, and AR were determined in the peritoneal fluid of patients with a NO , OVC, and OVCA. Heparin-binding epidermal growth factor levels were significantly enhanced in all stages in OVCA patients compared with levels in NO and OVC patients ($P<0.01$) (Figure 4A and Table 2). Transforming growth factor-α levels were quite low (less than 40 pg ml^{-1}) in all cases, and 46 out of 99 cases (four cases of NO, six cases of OVC, 30 cases of OVCA, and six cases of recurrence of OVCA) showed levels lower than the detection limit (5 pg ml^{-1}). Amphiregulin levels were scattered among the cases, but were less than 1000 pg ml^{-1} except in three OVCA cases. No significant differences were found in TGF-α and AR levels among the patients with NO, OVC, and OVCA (Figure 4B, C and Table 2). In addition to the significant increase of HB-EGF levels in OVCA patients, the concentration of HB-EGF in the peritoneal fluid of OVCA patients was much higher than those of TGF-α and AR (Figure 4), suggesting that HB-EGF is a major EGF family ligand, and is involved in tumour growth and OVCA extension.

To elucidate the relationship between EGFR ligands and clinical outcome, the amounts of EGFR ligands in peritoneal fluid were compared between before and after chemotherapy. Of the six cases examined, three cases showed a good response to neo-adjuvant chemotherapy and a marked reduction in tumour size and peritoneal fluid. In these cases, computed tomography indicated the disappearance of the tumour in the abdomen with six courses of chemotherapy. The other three cases did not respond to neo-adjuvant chemotherapy and there was no marked effect on tumour growth or the volume of peritoneal fluid. The levels of HB-EGF were dramatically reduced in the former cases, but were slightly increased in the latter ones (Figure 5A). No significant changes in TGF-α and AR were observed between pre- and post-chemotherapy (Figure 5B and C). These results suggest that HB-EGF levels in peritoneal fluid might reflect the response to chemotherapy.

DISCUSSION

In this study, we have shown the following: (1) HB-EGF levels in peritoneal fluid were elevated at all stages and at recurrence, and levels of HB-EGF were sufficient to proliferate and to allow survival of OVCA cells, whereas the levels of the other five EGFR ligands in peritoneal fluid had little effect on the proliferation of OVCA cells. (2) Cell proliferation of OVCA cells was stimulated by the addition of HB-EGF, but not LPA, in *in vitro* study. (3) Changes in the amount of HB-EGF were reflected by therapeutic efficacy in OVCA patients. Taken together, the present study suggests that HB-EGF plays a pivotal role in tumour growth and extension of OVCA. In this study, however, cell proliferative activities in SKOV3, RMG-1, or OVMG1 cells, which were mediated by peritoneal fluid from OVCA patients, were not always dependent on the expression of EGFR or ErbB-4. It remains open to debate how EGFR or ErbB-4 is involved in the cell proliferation mediated by peritoneal fluid in OVCA patients.

Among the members of EGF family growth factors, HB-EGF, AR, and betacellulin have heparin-binding properties (Raab and Klagsbrun, 1997; Iwamoto and Mekada, 2000; Strachan *et al*, 2001). Heparin-binding epidermal growth factor is also known to have a broad spectrum of biological activities including mitogenic activity, chemotaxis, adhesion, and angiogenesis (Raab and Klagsbrun, 1997; Iwamoto and Mekada, 2000). Increasing evidence indicates that HB-EGF is involved in various pathophysiological disorders. Recently, it has been shown that EGFR is transactivated by a variety of stimuli through the ectodomain shedding of EGFR ligands, and that HB-EGF plays a central role in this process (Prenzel *et al*, 1999). Lysophophatidic acid and other ligands for G protein coupled receptors (GPCR) also transactivate EGFR through ectodomain shedding of HB-EGF or AR (Prenzel *et al*, 1999; Umata *et al*, 2001; Gschwind *et al*, 2003). In OVCA, LPA has been

Table 2 Concentrations of HB-EGF, TGF-α, and amphiregulin, and cell proliferation properties in peritoneal fluid

	Concentration of EGF ligands (pg ml^{-1}, mean ± s.d.)			Proliferation-promoting activities (mean ± s.d.)	
Variable	HB-EGF	TGF-α	Amphiregulin	[^{3}H]Thymidine incorporation (c.p.m.)	WST-1 assay (abs.)
Normal ovary ($N=18$)	483 ± 495	11.12 ± 13.01	98 ± 236	9216 ± 5777	0.12 ± 0.11
Ovarian cyst ($N=18$)	653 ± 616	7.37 ± 3.67	186 ± 322	8990 ± 5381	0.26 ± 0.18
Ovarian cancer, stage Ia ($N=10$)	3090 ± 2286*	7.45 ± 4.01	214 ± 183	21 747 ± 8678**	0.71 ± 0.29***
Ovarian cancer, stage Ic–II ($N=13$)	2132 ± 1394*	8.33 ± 7.47	203 ± 220	18 654 ± 4988**	0.76 ± 0.33***
Ovarian cancer, stage III–IV ($N=30$)	2053 ± 1204*	5.14 ± 0.62	225 ± 755	24 556 ± 10 152**	0.86 ± 0.66***
Ovarian cancer, Recurrence ($N=10$)	2544 ± 1098*	2.01 ± 5.12	212 ± 175	21 035 ± 9755**	0.78 ± 0.28***

*$P<0.01$ for comparison of HB-EGF level *vs* normal ovary. **$P<0.01$ for comparison of c.p.m. count *vs* normal ovary. ***$P<0.01$ for comparison of absorbance *vs* normal ovary.

Figure 1 Cell proliferation activity mediated by peritoneal fluid in patients with a normal ovary (NO), an ovarian cyst (OVC), or ovarian cancer (OVCA). (**A**) Expression of EGFR and ErbB-4 protein in SKOV3, RMG-1, and OVMG1 cells. Lanes 1 and 4: SKOV3 cells. Lanes 2 and 5: RMG-1 cells. Lanes 3 and 6: OVMG1 cells. (**B**) The value of absorbance in the WST-1 assay in SKOV3 cells incubated with patients' peritoneal fluid from an NO, an OVC, and an OVCA at clinical stages Ia, Ic-II, III–IV, and recurrence. Closed circles indicate the value of absorbance in each patient. Horizontal lines indicate mean values. The *P*-value represents comparison with the levels of patients with an NO and an OVC. (**C**) The [^{3}H]thymidine incorporation in SKOV3 cells incubated with patients' peritoneal fluid of a NO, an OVC, and an OVCA at clinical stages Ia, Ic-II, III–IV, and recurrence. Closed circles indicate the value of [^{3}H]thymidine incorporation in each patient. Horizontal lines indicate mean values. The *P*-value represents comparison with the levels of patients with a normal ovary and an ovarian cyst. (**D**) Alterations in the [^{3}H]thymidine incorporation of an ovarian cancer patient's peritoneal fluid by anti-EGFR ligand antibodies or an anti-EGFR antibody. A bar indicates the mean value and standard errors. The *P*-value represents comparison with the levels of patients in the absence of inhibitory antibodies.

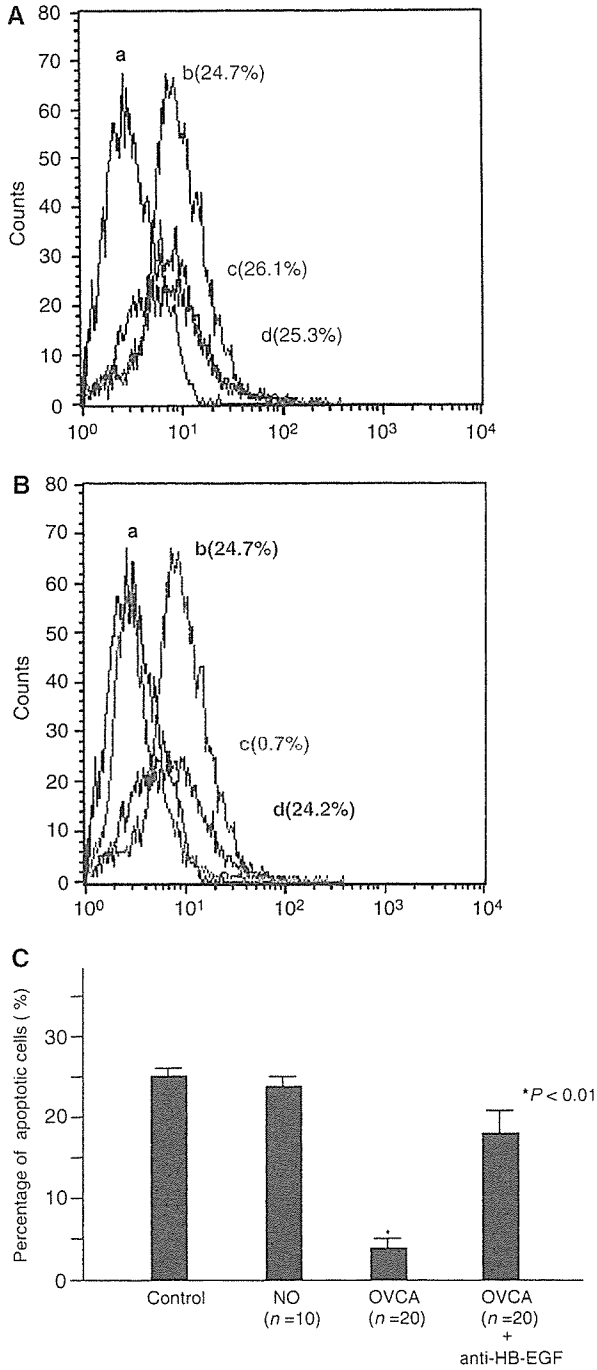


Figure 2 Cell survival activity mediated by peritoneal fluid in patients with a normal ovary or ovarian cancer. Flow cytometric analysis for apoptotic cells in SKOV3 cells after incubation with peritoneal fluid of a normal ovary (**A**) and ovarian cancer (**B**). Control (a: black line). Under serum-free condition (b: green line). Incubation with 10% peritoneal fluid in the absence (c: red line) or presence (d: blue line) of an inhibitory antibody against HB-EGF. Each percentage indicates the ratio of apoptotic cells in SKOV3 cells. (**C**) Alteration in the percentage of apoptotic cells after incubation with the peritoneal fluid from a normal ovary or ovarian cancer. A bar indicates the mean value and standard errors. The *P*-value represents comparison with the levels of patients with a normal ovary.

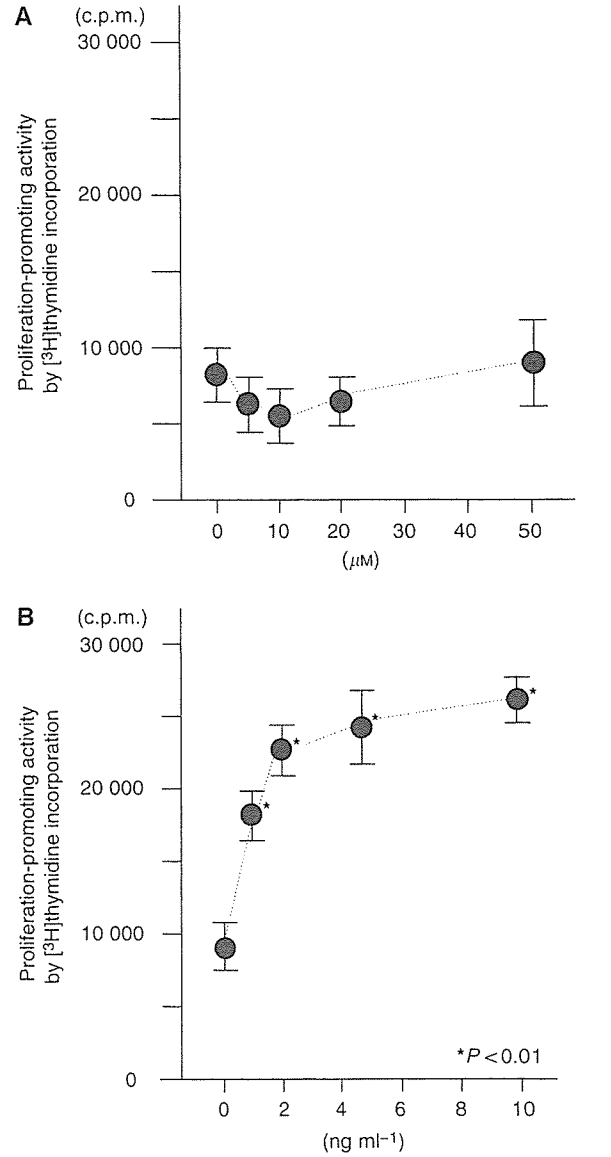


Figure 3 Mitogenic activity of LPA (**A**) and HB-EGF (**B**) in the presence of peritoneal fluid. Each point indicates the mean and standard deviation of [^{3}H]thymidine incorporation. The *P*-value represents comparison with the levels of [^{3}H]thymidine incorporation incubated in the medium containing peritoneal fluid of a patient with a normal ovary.

identified as a candidate for an OCAF (Xu *et al*, 1995; Westermann *et al*, 1998; Xiao *et al*, 2001). In our study, LPA did not stimulate SKOV3 cell proliferation in the presence of peritoneal fluid of NO patients; however, the effect of LPA might be dependent on the cell system. In contrast, HB-EGF stimulated SKOV3 cell proliferation in the same culture conditions. Therefore, it is likely that LPA is an OCAF, but it stimulates OVCA cell proliferation by inducing the ectodomain shedding of HB-EGF, rather than directly acting by itself on cancer cells.

In OVCA, LPA regulates the production of LPA itself through the activation of phospholipase D and phospholipase A2 (Eder *et al*, 2000). Similarly, HB-EGF induces HB-EGF gene expression itself by activating mitogenic properties (Tan *et al*, 1994). In addition, LPA and HB-EGF appear to influence their reciprocal production. Lysophophatidic acid can enhance

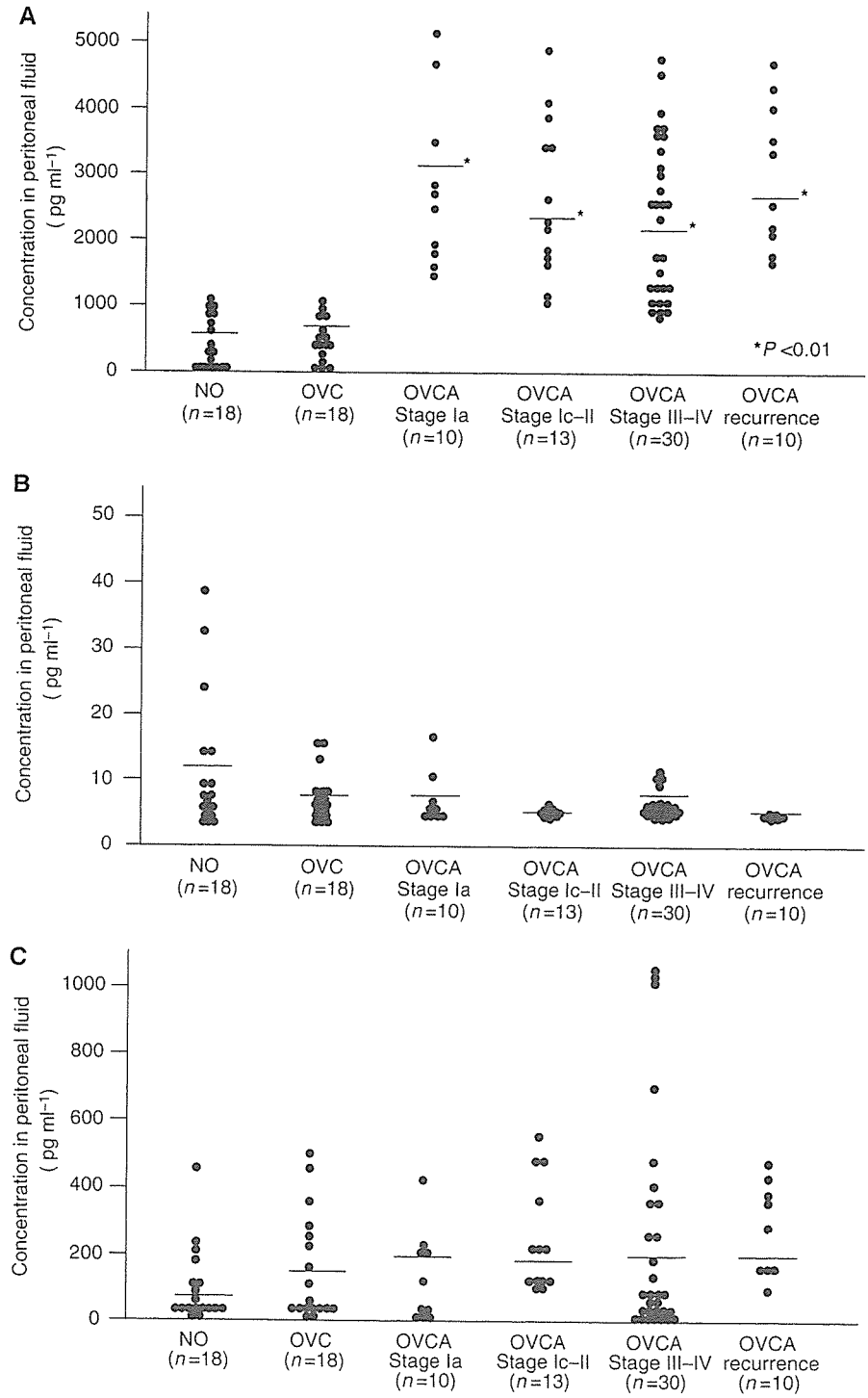


Figure 4 Distribution of HB-EGF (**A**), TGF-α (**B**), and AR (**C**) concentration in peritoneal fluid among patients with a normal ovary (NO), an ovarian cyst (OVC), or ovarian cancer (OVCA) at clinical stages Ia, Ic-II, III–IV, and recurrence. Closed circles indicate the value of EGFR ligand concentration in each patient. Horizontal lines indicate mean values. The *P*-value represents comparison with the levels of patients with an NO or an OVC.

the gene expression of HB-EGF (Goetzl *et al*, 1999), as well as induce the ectodomain shedding of HB-EGF (Prenzel *et al*, 1999; Umata *et al*, 2001). Heparin-binding epidermal growth factor activates EGFR, and the activated EGFR enhances the activity of phospholipase D, which in turn causes the production of LPA (Yeo and Exton, 1995). Considering this evidence as a basis, HB-EGF and LPA might collaborate in inducing cell proliferation and in amplifying their own production. In this study, we showed that HB-EGF is elevated in the ascitic fluid of OVCA patients even at an early stage. Lysophophatidic acid may contribute to the production of HB-EGF, especially in an early phase of OVCA.

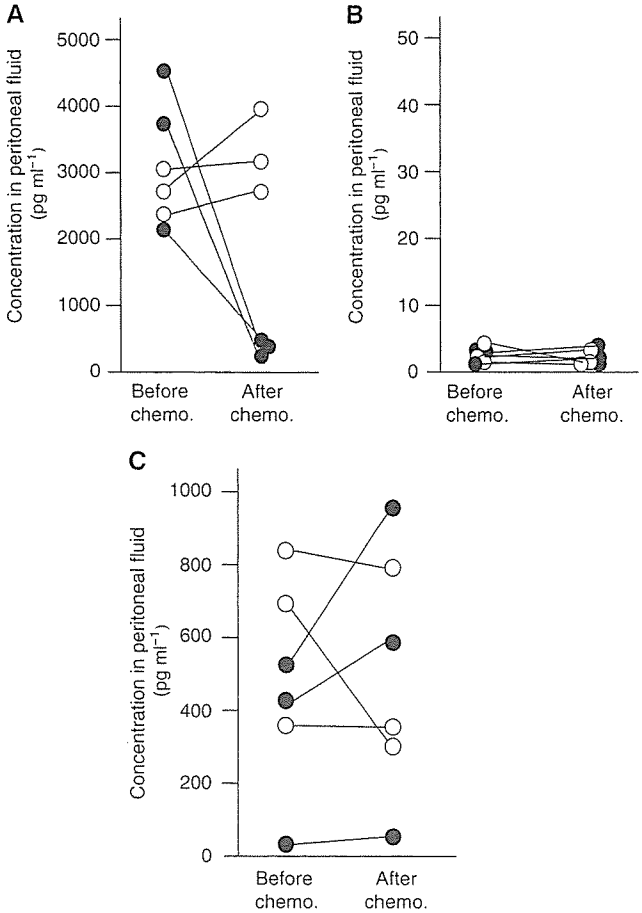


Figure 5 Changes in concentrations of HB-EGF (**A**), TGF-α (**B**), and AR (**C**) in the peritoneal fluid among patients with ovarian cancer between pre- and post-chemotherapy. Open circles indicate the concentration of each peritoneal fluid from chemotherapy-non-responding patients. Closed circles indicate the concentration of each pentoneal fluid from chemotherapy-responding patients.

Heparin-binding epidermal growth factor in peritoneal fluid is generated mainly from cancer cells and peritoneal mesothelial cells, which have the same origin as coelomic epithelium of the NO. Our study showed that HB-EGF levels were already elevated even in the ascitic fluid of patients with stage Ia OVCA, whereas OVCA cells are enclosed within the cyst wall. This suggests that HB-EGF is not secreted from cancer tissues in an early stage. In peritoneal mesothelial cells, HB-EGF is constitutively expressed and produced by cytokine stimulation (Jayne *et al*, 2000). Therefore, it is possible to speculate that during early OVCA, mainly peritoneal mesothelial cells produce HB-EGF in the peritoneal fluid by stimulation of a variety of cytokines or by LPA. In advanced stages of OVCA, however, the HB-EGF levels in the peritoneal fluid appear to be correlated with the tumour state in the peritoneal cavity, as HB-EGF levels of patients' fluids were largely reduced after chemotherapy in chemotherapy-responding cases. Therefore, in advanced OVCA, both cancer and peritoneal mesothelial cells might produce HB-EGF.

These results are the first demonstration of HB-EGF as an OCAF. Heparin-binding epidermal growth factor is known to enhance cell motility as well as cell proliferation. Therefore, elevated HB-EGF in peritoneal fluid may contribute to not only survival and proliferation of cancer cells, but also to their dissemination into the peritoneal cavity. It is easier to measure HB-EGF levels in peritoneal fluid compared with LPA, suggesting that HB-EGF is a promising bioactive marker of OVCA. For therapy, relevant LPA antagonists have yet to be developed, while Iressa, a specific EGFR tyrosine-kinase inhibitor, is not effective in OVCA (Baselga *et al*, 2002). Thus, the development of therapeutic tools against HB-EGF would allow the exploration of novel targeting therapies for OVCA.

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