

Osteosarcoma: Current status of immunotherapy and future trends (Review)

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Received September 9, 2005; Accepted November 16, 2005

Abstract. Osteosarcoma is the most common primary bone tumor and represents a major therapeutic challenge in medical oncology. While the use of aggressive chemotherapy has drastically improved the prognosis of the patients with non-metastatic osteosarcomas, the very poor prognosis of patients with metastasis have led to the exploration of new, more effective and less toxic treatments, such as immunotherapy for curing osteosarcoma. Compared to the numerous reports describing successful immunotherapy for other solid tumors, the number of reports concerning immunotherapy for osteosarcoma is low. However, this therapeutic strategy opens new areas for the treatment of osteosarcoma. In this review, the reasons for delay and all elements essential to develop immunotherapy concerning osteosarcoma are defined. Several pieces of evidence strongly support the potential capability of new therapies such as cellular therapy and gene therapy to eradicate osteosarcoma. Thus, clinical human trials using peptides, cytokines and dendritic cells have been performed. Tumor-infiltrating lymphocytes and some tumor antigens have been identified in osteosarcoma and resulted in an important breakthrough in cellular immunotherapy. Also, RANKL/RANK/OPG, the key regulator of bone metabolism, is a hot spot in this field as therapeutic tools. Immunotherapy for osteosarcomas has great potential, promising improvement in the survival rate and better quality of life for the patients.

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1. Introduction

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Key words: osteosarcoma, immunotherapy, bone metabolism, apoptosis

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1. Introduction

Osteosarcoma, the most frequent primary bone tumor, typically affects children and young adults (1). The overall survival with an aggressive chemotherapy regimen before and after surgery now varies between 50 and 65% (2). These poor results have led to the exploration of new, more effective and less toxic treatments, such as immunotherapy for curing osteosarcoma.

Immunotherapy is a therapeutic strategy based on the up-regulation of the immune response in tumor-bearing hosts. Two immunotherapy types exist: i) passive or adoptive immunotherapies consist of the administration of *ex vivo*-expanded tumor-specific cytotoxic immune cells especially T lymphocytes, ii) active immunotherapies including pulsed dendritic cells and cytokine treatments that elicit immune response against tumor cells. Several reports have strongly underlined the potential interest of these new therapies applicable to osteosarcomas. We summarized the pathway of the representative immunotherapy for osteosarcoma in theme (Fig. 1). This review focuses on the current knowledge as well as the future trends of immunotherapies for osteosarcomas.

2. Therapeutic strategies based on passive immunity also named adoptive immunity

Cytotoxic T lymphocytes (CTL) specifically recognizing tumor cells are the pivot cells of passive immunotherapies. Monoclonal, polyclonal and cell lines of T lymphocytes have already been envisaged to develop such therapeutic strategies.

Tumor antigens inducing HLA class I-restricted tumor-specific cytotoxic T lymphocytes (CTL). The identification of human cancer antigens restricted to HLA class I opened a new area of antigen-specific cancer immunotherapy

specifically targeting these antigens (3). Specific immunotherapies utilizing peptides deriving from these antigens are ongoing for the treatment of HLA-A1+ patients suffering from melanoma and resulted in major clinical responses (4,5). Based on these observations, this therapeutic strategy was extended to other malignant tumors including osteosarcoma. Indeed, several tumor antigens: melanoma-associated antigen (MAGE) (6), squamous cell carcinoma antigen recognized by T cells (SART) 1 (7), SART3 (8) and papillomavirus binding factor (9) are expressed in osteosarcoma and provided the rationale to develop cellular therapies in osteosarcoma. A newly defined tumor-rejection antigen SART3 is highly expressed in osteosarcoma (8). SART3 was identified from esophageal cancer cells KE4 (10). The SART3-derived peptides were able to induce HLA-A2-restricted and tumor-specific CTL in various histological types (squamous cell carcinoma, astrocytoma and adenocarcinoma) (11). These facts support the potential use of the SART3-derived peptides for specific immunotherapy of HLA-A2+ patients suffering from osteosarcoma. SART3-derived peptides induce the production of SART3-specific CTL in an HLA-A24-restricted manner in osteosarcoma (8). Taken together with the prevalence of HLA-A24 (12), this strategy could be applicable for ~60% of HLA-A24+ patients with osteosarcoma. Furthermore, no severe adverse response associated with peptide administration and a significant up-modulation of the cellular immune response against tumor cells in clinical trial using SART3-derived peptides in HLA-A24+ patients with colon cancer (13) encourages further application of this strategy for osteosarcoma.

Polyclonal tumor-infiltrating lymphocytes (TIL): Selected immunotherapeutic weapon that directly induces apoptosis in cancer cells. An immunohistochemical study revealed infiltration of osteosarcomas by T lymphocytes (14). Phenotypic analyses demonstrated that these infiltrating lymphocytes were 95% CD3+ and 68% CD8+ (14). Rivoltini *et al* have also performed phenotypic analyses of TIL in 37 pediatric tumors, including 12 osteosarcomas and revealed their CD8+ predominancy (15). It is theorized that the infiltrating lymphoid represents a selected population of cells which have preferentially migrated to the tumor secondary to an immune response. These T lymphocytes termed TIL are considered to be more specific in their immunological reactivity to tumor cells than the non-infiltrating lymphocytes (16). Thus, the identification of tumor-specific lymphocytes has resulted in new therapeutic strategies based on mounting a sustained and effective anti-tumor immune response (16,17). Recently, we have shown that only TIL extracted from osteosarcoma were cytotoxic against allogeneic tumor cells in the analyses of 27 human patients with bone-associated tumors (osteosarcoma, Ewing's sarcoma, giant cell tumor, chondrosarcoma, plasmocytoma and bone metastases) (18). Furthermore, TIL lytic activity was significantly higher compared to autologous peripheral blood leukocytes. Moreover, TIL extracted from rat osteosarcoma were very sensitive to the tumor antigens expressed by autologous tumor cells and demonstrated increased proliferation (18). These findings strongly support the potential capability of TIL therapy for osteosarcoma.

Rivoltini *et al* reported in 1992, that TIL obtained from pediatric patients were difficult to use for immunotherapy at required levels (15); however, recent *in vitro* culture methods have shown great advances. Now, one of the most important conditions of T cell immunotherapy is their anergic/tolerant manner against tumor cells (19). It has been reported that the Fas-mediated apoptosis pathway plays a crucial role in this condition (19,20); however these poor immune responses could be normalized upon *in vitro* culture (21,22). Furthermore, immunotherapy combinations with chemotherapeutic agents induce an anti-tumor effect for Fas-mediated apoptosis resistant tumors (23-25). Moreover, interferon (IFN)- γ sensitizes osteosarcoma cells to Fas-induced apoptosis through up-regulation of the Fas receptor (26). Combined immunotherapy with IFN- γ and either anti-Fas monoclonal antibody or CTL bearing Fas ligand (FasL) might be useful. Thus, TIL remain a viable arm of immunotherapy for osteosarcoma similar to clinical phase II trials in melanoma (27,28).

Except for tumor immune escape in osteosarcoma, the Fas/FasL pathway plays a crucial role in chemotherapy-induced apoptosis (25) and metastasis (26,29,30). Thus, this pathway was used as a therapeutic target in several strategies (31,32). Mainly, osteosarcoma patients die from lung metastasis; therefore Fas/FasL may be a good therapeutic target, especially as a lung metastasis inhibitor.

Another important factor of T cell therapy is the immunological specificity of T cells for the tumor (19,33). One approach is to use *ex vivo*-expanded T cell clones demonstrating specific lysis of an antigen-positive tumor target. As shown in phase-I study in metastatic melanoma, several advantages of T cell clone strategy were demonstrated without severe toxic side effects (5,34,35). This T cell clone strategy will be able to achieve more effective and less toxic T cell therapy for osteosarcoma.

Natural killer (NK) cells and T cell lines: The cell populations specifically directed against tumor cells. NK cells have innate anti-tumor functions upon tumor regression (36). TALL-104 is endowed with MHC non-restricted killer activity against a broad range of tumors across several species, sparing cells from normal tissues (37). TALL-104 cells were administered systemically in an adjuvant setting to 23 cases of canine osteosarcoma after surgery and chemotherapy (38). This therapy achieved favorable median survival times and disease-free intervals compared with canine osteosarcoma treated with standard therapy, and supported the efficacy of adjuvant TALL-104 cell administration. In this series, severe side effects including TALL-104 cell-induced leukemia were not observed, thus this strategy could be worthwhile also in humans.

To up-regulate NK cell-mediated anti-tumor function, certain strategies have been envisaged (cytokines are mentioned below). Kubista *et al* reported that hyperthermia increases the susceptibility of osteosarcoma cells to NK-mediated lysis by increased expression of heat shock proteins (hsp) 72 (39). Hsp 72, implicated in tumor immunity (40), is involved in the interaction between T lymphocytes and hsp72+ osteosarcoma cells (41).

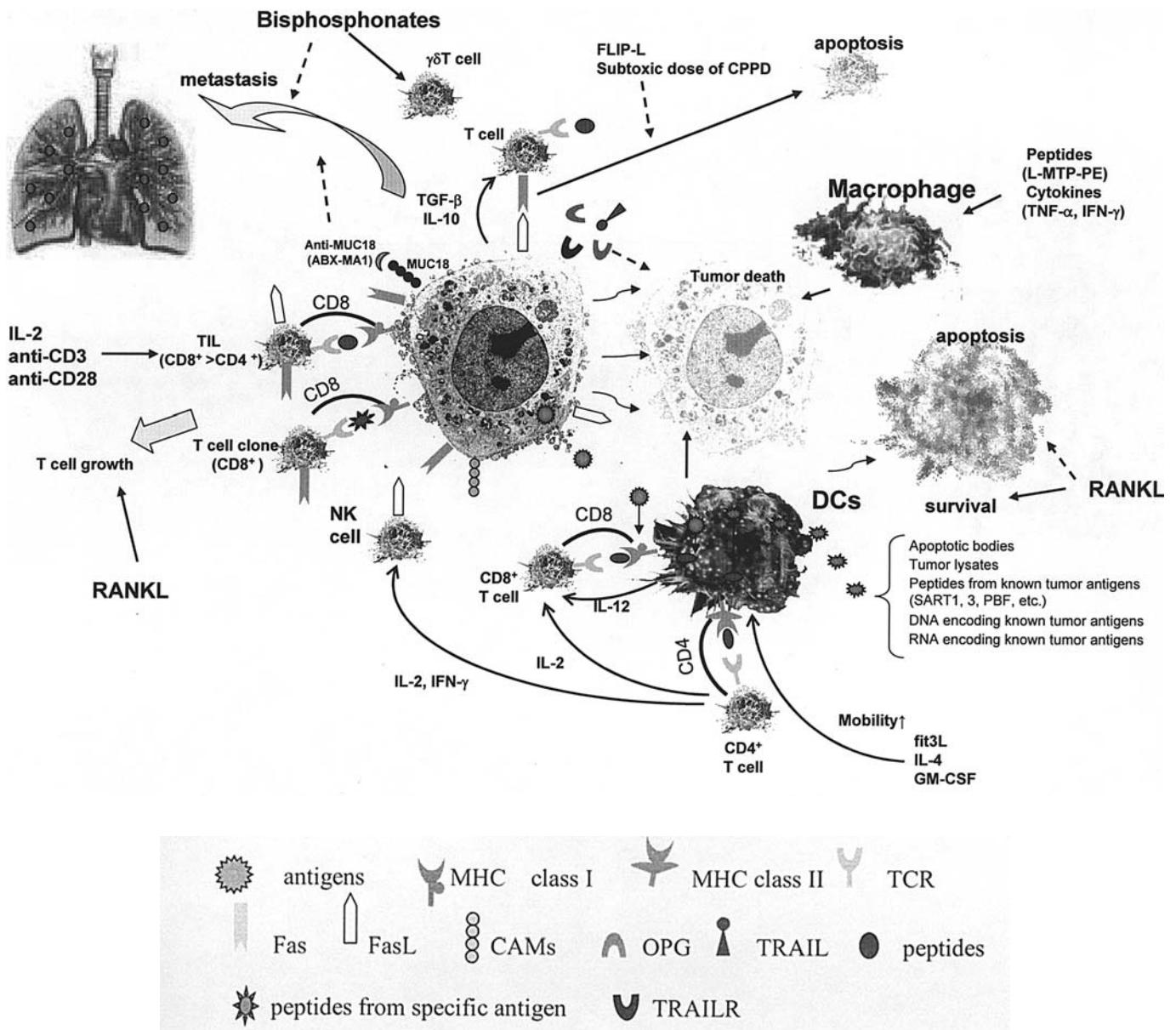


Figure 1. Potential immunotherapeutic approaches against osteosarcoma cells. T cells such as TIL and NK cells directly attack tumor cells in an MHC-restricted manner or not, according to their populations. Administration of DCs induces up-regulation of T cells by presenting peptides in an MHC-restricted manner and also directly targets tumor cells. For priming DCs, several strategies were identified. Cytokine networks stimulate immune therapeutic cells. Administration of peptides (L-MTP-PE) and cytokines (TNF- α , IFN- γ) stimulate macrophages. Bisphosphonates have potential anti-tumor effects as metastasis inhibitors and modulate immune response as $\gamma\delta$ T cell activators. RANKL cannot only prolong the survival time of DCs, but also induce T cell growth. OPG acts as a decoy receptor of TRIL. CAMs, cell adhesion molecules; CDDP, cisplatin; DCs, dendritic cells; fit3L, fit3 ligand; FLIP-L, FLICE inhibitory protein long form; FasL, Fas ligand; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; L-MTP-PE, liposome-encapsulated muramyl tripeptide phosphatidylethanolamine; RANKL, receptor activator of nuclear factor- κ B ligand; OPG, osteoprotegerin; PBF, papillomavirus binding factor; SART1, 3, squamous cell carcinoma antigen recognized by T cells 1, 3; TCR, T cell receptor; TGF- β , transforming growth factor- β ; TIL, tumor-infiltrating lymphocytes; TNF- α , tumor necrosis factor- α ; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor; black solid arrow, activation; dotted arrow, suppression.

3. Therapeutic strategies based on active immunity

To elicit immunity of tumor-bearing hosts, antigen presenting cells (APCs) such as dendritic cell (DC)-based therapies, cytokine-based therapies and gene therapies have been demonstrated.

Monocyte lineage constitutes a complex system of professional APCs which can induce primary T and B cell responses. DCs constitute a complex system of professional APCs that have the unique capacity to induce primary T and B cell responses

(42). The main pathway of DC-based immunotherapy is to up-regulate lymphocyte activity, such as NK cells and TIL, and the goal will be to optimize the use of DCs (i.e., vaccination) in maintaining T lymphocyte survival and specificity. A number of clinical trials are currently underway studying DCs in a variety of tumors (43). One clinical phase-I study using DCs against solid tumors in children including osteosarcoma has been reported (44). In this series, one patient with metastatic fibrosarcoma demonstrated strong positive response without obvious toxic side effects. Some relevant topics include antigen loading and DC maturation procedures,

frequency and route of DC administration, efficacy of DCs homing to lymphoid tissues and their durability once there and the role of distinct DC subsets (42,43).

Monocyte/macrophage-mediated tumor cell killing is a major mechanism of the hosts' defense against primary and/or metastatic neoplasms. Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) is a peptide that acts as a potent activator of monocytes/macrophages in humans, mice and dogs (tumor antigen-derived peptides used to produce T cells *in vitro* or to charge DCs). In over 125 dogs with osteosarcoma, L-MTP-PE was found to prolong metastasis-free and overall survival rates when given alone or after systemic chemotherapy (45,46). Kurzman *et al* reported that canine pulmonary alveolar macrophages from dogs treated with doxorubicin (DOX) + L-MTP-PE have enhanced cytotoxic activity against osteosarcoma cells when compared to dogs treated with DOX or L-MTP-PE alone (47). These findings support the rationale for combining chemotherapy agents with immunotherapy for the treatment of metastatic disease. The greater anti-tumor activity of L-MTP-PE has been also demonstrated in children with metastatic osteosarcoma and a phase-III randomized trial has been started (48,49).

Cytokines: most widely used and investigated, possibly essential molecules in immunotherapy due to their excellent wide range ability. Cytokines represented by interleukins (ILs) play a crucial role in the expression of cellular adhesion molecules (CAMs) and the function of anti-tumor effector cells as the most potent modulators of the immune responses. CAMs play an important role in immune responses including NK cell binding to target (50). Indeed, melanoma CAM, synonymous MUC18 plays a crucial role in osteosarcoma metastasis (51). Osteosarcoma cells express this molecule and ABX-MA1, a fully human anti-MUC18 antibody, inhibited the metastasis of human osteosarcoma cells *in vivo* (51). The most widely studied IL in this field is IL-2 (52). Luksch *et al* have reported a clinical trial in osteosarcoma using IL-2 (53), in which 18 children with localized osteosarcoma received four IL-2 courses (9×10^6 IU/ml/day $\times 4$), alternated with pre- and post-operative multiple chemotherapies. The results showed that intensive chemotherapies have no effect on the IL-2-induced immune activation, and suggested a role of the NK cells in the control of osteosarcoma. On the contrary, it has been reported that the clinical use of IL-2 is limited by the significant toxic side effects caused by the administration of this cytokine in doses sufficient for cell activation *in vivo* (54). Other ILs have been recognized as candidates for human immunotherapy. Some studies using IL-12 (55), IL-12 associated with IL-18 (56), IL-18 (57) and IL-17 (58) in osteosarcoma have already been performed. These cytokine-based therapies demonstrated enhanced cytotoxic activity of T cells in osteosarcoma. Also, it has been reported that tumor necrosis factor (TNF)- α and IFN- γ can induce the anti-tumor activity of macrophages (47).

Gene therapy eliciting immune response in tumor-bearing hosts represents one option of immunotherapy. In the field of gene therapy for osteosarcoma, several approaches have been envisaged, such as the suicide gene therapy (59,60), tumor-

suppressor gene therapy (61-64) and cytokine-based gene therapy (65-68). The most investigated gene transfer vector is the adenoviral vector (Adv) (69). A single injection of Adv-encoding IL-2 gene (Ad IL-2) into a primary tumor lesion elicited anti-tumoral immunity and this immunity not only suppressed primary tumor growth but also eradicated disseminated micro-metastases in distant organs (70). In this study, not only minimal side effects but also maximal therapeutic effects were exerted only in the case of injecting the optimal dose (not the highest) of Ad IL-2. Important limitations in this regard are the failure of non-replicating Adv to achieve sufficient tumor-cell transduction and effective solid-tumor penetration. Furthermore, the expression of coxackievirus and adenovirus receptor, which is an important determining factor for adenoviral gene transfer efficiency, in osteosarcoma is controversial (71-73). Witlox *et al* demonstrated that targeting a conditionally replicative adenovirus toward integrins Ad5- $\Delta 24$ RGD, providing alternative viral entry pathway, greatly enhances its cytotoxicity on osteosarcoma and warrants further exploration of Ad5- $\Delta 24$ RGD for its utility in osteosarcoma treatment (74). However, the fetal case report following adenovirus gene transfer (75) indicated against this strategy in humans. As other than adenovirus gene transfer vectors, it has been shown that osteosarcoma cell lines were good targets for lentiviral transduction with favorable gene transfer efficiency (76,77). After the development of a successful and safe delivery of the therapeutic gene, this strategy demonstrates great potential activity to modulate the prognosis of patients with osteosarcoma.

4. Summary and future trends

There is no doubt that one of the most significant advances in the field of anti-cancer therapy has been the recent development of immunotherapy; however, the initial results of human trials were not realized as expected. The reasons for this discrepancy have been reported (19,78). It is now common knowledge that the tumor burden contributes to a significant suppressive environment. Thus, surgery remains the first line for debulking tumors and radiation and/or chemotherapy can be used for the removal of remaining and micro-metastatic lesions as well as reducing tumor burden. To achieve desired results, immunotherapies combined with these conventional treatments are recommended.

The number of published data of immunotherapy for bone tumors is very low compared with that of other solid tumors. The reasons for this delay were discussed (9,79) and the following points were raised; i) the relatively low immunogenicity of osteosarcoma as only few examples of spontaneous tumor regression exist (80,81), ii) the practical difficulty in establishing osteosarcoma cell lines and autologous CTL (82,83) and iii) the lack of suitable candidate genes for a reverse immunological approach such as a tumor-specific fusion gene (84,85). However, another hopeful explanation of this delay resides in environmental factors peculiar to bone. Recent studies have clarified molecules, such as the receptor activator of nuclear factor- κ B ligand (RANKL)/RANK/osteoprotegerin (OPG) as the key regulators of normal and pathological bone metabolism (86-90).

Thus, correlations between the phenotypes of the tumors and changes of RANKL/OPG have been reported (91). In osteosarcoma, high OPG (92,93) and lack of RANKL at the mRNA level (94) have been reported. To prevent bone destruction due to malignancies, the potential capability of these molecules as therapeutic tools has been suggested (95). As osteoclast is the unique cell that can induce bone degradation, inactivation of the osteoclast by OPG was targeted. Honore *et al* have reported that the administration of OPG blocked bone cancer-induced skeletal destruction (96). Furthermore, direct effects of RANKL/RANK/OPG on immune response were reported. Specifically, RANKL can dramatically inhibit DC apoptosis via increased Bcl-xL expression (97) and induce T cell growth (98). OPG acts as a weak decoy receptor for TNF-related apoptosis-inducing ligand (TRAIL) (99) and modulates tumor apoptosis (100). Also, bisphosphonates (BPs) can be another therapeutic approach for osteosarcoma. Except for known function of BPs, the inhibitory effects of BPs on the metastases as well as the potent anti-cancer effect have been suggested (101). Moreover, as BPs can activate $\gamma\delta$ T cells involved in tumor cell surveillance and killing (102), the ability of BPs as $\gamma\delta$ T cell activators is encouraging for immunotherapy. These results provide the rationale to use the molecules in immunotherapy for osteosarcoma; however, the safe administration of these agents in humans should be addressed carefully.

5. Conclusion

To date, the number of published clinical trial of immunotherapy for osteosarcoma is low. However, there are several pieces of evidence strongly supporting the potential capability of immunotherapy to eradicate osteosarcoma in combination with conventional treatment. Immunotherapy for osteosarcomas has potential promising improvement in the survival rate and better quality of life for patients with this tumor.

Acknowledgements

This study was partially supported by grants from Uehara Memorial Foundation and Kanae Foundation for Life & Socio-Medical Science, and also supported by a grant from the Association pour la Recherche contre le Cancer (ARC) No. 3561.

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