American Medical Journal 3 (2): 240-248, 2012
ISSN 1949-0070

The Role of Inflammation in Development and Therapy of Malignant Mesothelioma

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Abstract: Malignant Mesothelioma (MM) is an asbestos related malignancy with a poor prognosis and limited therapeutic approaches. The pathogenesis of MM has been linked to asbestos induced inflammation. Asbestos exposure results in reactive oxygen species generation, infiltration of inflammatory cells and prolonged release of multiple cytokines, oxidants and growth factors. The role of inflammation in MM has led to the evaluation of inflammatory profiles as prognostic and therapeutic markers. Additionally, inflammatory pathways are under investigation for potential therapeutic interventions. In this review, we discuss the role of inflammation in MM pathogenesis, inflammatory markers with potential clinical impact for MM and clinical trials that target inflammatory pathways and responses for treatment of MM. Ultimately, MM remains a difficult to treat cancer that requires multimodality therapy.

Key words: Best Investigator's Choice, Malignant Mesothelioma (MM), Reactive Oxygen Species, Malignant Pleural Mesothelioma, Extrapleural Pneumonectomy

INTRODUCTION

Malignant Mesothelioma (MM) is caused by asbestos and produces devastating tumor types with poor prognosis and no effective therapeutic approaches. Asbestos exposure is known to increase the risk of pulmonary pathology in the form of nonmalignant inflammatory diseases, such as pleural plaques, pleural effusions and asbestosis and malignant diseases such as mesothelioma and bronchogenic carcinoma. The differences in the cellular phenotype involved (alveolar, epithelial, or mesothelial cells) defines the outcome of disease. Asbestos-induced inflammatory changes may, in part, be responsible for diseases. Pleural plaque is produced by the effect of recurrent inflammatory and repair processes occurring for long time periods. Chronic inflammatory episodes may predispose to malignant evolution, as it is known that the majority of MM develops on pleura affected by pleural plaques and not on the normal pleura. A survey of the current literature suggests that there is more than one mechanism involved in the pathogenesis of asbestos-induced mesothelioma. One proposed mechanism is the oxidative stress concept that highlights how iron within asbestos fibers catalyzes free radical generation and thereby induces oxidative stress and carcinogenesis (Shukla *et al*., 2003). Another proposed mechanism is the chronic inflammatory theory that is founded on the observation that inflammation is a biologic response to pathogenic materials and injured cells in which various types of cells, such as neutrophils, macrophages, fibroblasts and vascular endothelial cells, interact with each other. Over time, inflammation becomes chronic and plays an important role in asbestos-induced carcinogenesis that is characterized by persistent release of cytokines and oxidants from macrophages. One way proinflammatory cytokines promote carcinogenesis in epithelial and mesothelial cells is by altering signaling pathways and thereby inhibiting apoptosis. In addition, persistent macrophage activation plays important roles in initiation, as well as promotion, of pathological processes in mesothelioma. The remaining mechanisms include the chromosome tangling concept, which postulates that asbestos fibers damage chromosomes when cells divide and the adsorption concept which states that the surface of asbestos possesses a high affinity for certain molecules including components of cigarette smoke as well as endogenous molecules. In the present review, we focus on the role of inflammation in development and therapy of MM.

Asbestos exposure related inflammatory changes leading to malignant mesothelioma: Asbestos exposure to cells and tissues results in Reactive Oxygen Species (ROS) generation that triggers lipid peroxidation, leading to inflammation related gene

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expression (Janssen-Heininger *et al*., 2000; Shukla *et al*., 2003). Our group has repeatedly demonstrated the role of asbestos in inflammation. Using mouse asbestos-inhalation models, we have shown inflammation in terms of altered levels of cytokines, differential cell counts in bronchoalveolar lavage fluid (BALF) (Sabo-Attwood *et al*., 2005; Shukla *et al*., 2007) and myeloperoxidase status in lung neutrophils (Haegens *et al*., 2005). In addition, Ogami *et al*. (2004) demonstrated an asbestos-induced inflammatory condition by morphometric analysis of rat lungs. In their experiment, rats were exposed to an asbestos dose comparable to asbestos levels detected in work environments (Ogami *et al*., 2004). It has been reported by many groups that the highest level of inflammation likelyoccurs at the site of fiber deposition. Pleural mesothelium function is possibly altered either directly or indirectly by the exposure of the lung to asbestos (Adamson, 1997). The growth phase of mesothelial cells in visceral pleura is very early and unrelated to pleural fiber deposition in an animal model of asbestos exposure. This process was found to depend on cytokine release by cells in the lung.

Although acute inflammatory responses in the lung parenchyma constitute an important defense mechanism in normal circumstances, chronic inflammation is considered to be an important contributor to tissue damage, inducing lung pathologies including mesothelioma. Fiber-induced inflammation in the parenchyma reverses both the normal flow of lymph and the normal transpleural pressure, resulting in a net flow of fluid and fibers directly into the pleural space from the underlying parenchyma (Miserocchi, 1997). This leads to mesothelial and endothelial cell damage, inflammation and the accumulation of pleural macrophages. Pleural macrophages undergo frustrated phagocytosis in an attempt to enclose the long fibers. The process of frustrated phagocytos is induces prolonged release of cytokines and oxidants that ultimately lead to further inflammation, fibrosis and genotoxicity in bystander mesothelial cells. Increased pathogenicity of long asbestos fibers depends on the persistent presence of fibers, repeated fiber-induced injury, tissue repair and local inflammation (Moalli *et al*., 1987; Donaldson *et al*., 1989).

As discussed below, there are numerous studies supporting the concept that there is an inflammatory reaction following asbestos exposure (Choe *et al*., 1997). Hill *et al*. (2003) reported that amosite asbestos causes pleural inflammation by increased secretion of Intercellular Adhesion Molecule-1 (ICAM-1), Monocyte Chemoattractant Protein-1 (MCP-1) and Macrophage Inhibitory Protein-2 (MIP-2) in pleural lavage fluid as well as in vitro mesothelial cell culture (Hill *et al*., 2003). Recent studies from our group have also shown that human mesothelial cells acquire resistance to asbestos-induced toxicity via induction of one or more Epidermal Growth Factor Receptor (EGFR)-linked survival pathways (PI3K/AKT/ERK1/2) during Simian Virus 40 (SV40) transformation and carcinogenesis (Shukla *et al*., 2011). Many cytokines and growth factors are shown to be implicated in asbestos-induced MM pathogenesis including Tumor Necrosis Factor alpha (TNF-α), Transforming Growth Factor Beta (TGF-β), Platelet Derived Growth Factor (PDGF), Insulin like Growth Factor (IGF) (Liu and Klominek, 2004), interleukin-6 (IL-6), interleukin-8 (IL-8) (Galffy *et al*., 1999), Vascular Endothelial Growth Factor (VEGF) (Strizzi *et al*., 2001) and Hepatocyte Growth Factor (HGF) (Cacciotti *et al*., 2001). TNF-α is released in response to large accumulations of macrophages undergoing phagocytosis of asbestos. The binding of the released TNF-α to its receptor, TNF-R1, which is also expressed by mesothelial cells and activated by NFκB pathway, increases the percentage of human mesothelial cells that survive asbestos exposure (Yang *et al*., 2006). Our recent in vitro and in vivo studies also support the role of asbestos in inflammation-induced mesothelioma. We recently reported, using Affymetrix microarray and BioPlex analysis in primary and telomerase immortalized human mesothelial cells, that asbestos exposure causes increased inflammatory responses that include IL-1β, IL-13, basic Fibroblast Growth Factor (bFGF), VEGF and granulocyte colony stimulating factor (G-CSF) which may be responsible for mesotheliomagenic transformation of these cells (Shukla *et al*., 2009; Hillegass *et al*., 2010a). In addition, our in vivo work confirms that MM development in intraperitoneal mouse models is preceded by increased levels of many of these cytokines and growth factors (Hillegass *et al*., 2010b).

Elegant work by our group in collaboration with others has demonstrated, for the first time, that the NOD-Like Receptor Protein 3 (NLRP3) inflammasome plays a critical role in asbestos inhalation-induced inflammation in mouse lungs (Dostert *et al*., 2008). Further studies in human mesothelial cells confirmed that asbestos exposure caused priming of NLRP3 (increased mRNA levels) as well as its activation (Shukla *et al*., 2003; 2007; 2009; 2011). In support of our work, Yang *et al*. (2010) showed the release of high mobility group box-1 protein (HMGB-1) from asbestos-exposed mesothelial cells (Yang *et al*., 2010). The release of HMGB-1 may possibly occur via inflammasome-induced pyroptosis, an inflammation-

mediated cell death process dependent on inflammsome activation of caspase-1. Additionally, a recent study has reported the ability of the iron in Libby amphibole to induce the inflammasome cascade (Shannahan *et al*., 2012).

Radiation can also induce mesothelioma in animals and humans and may be linked to inflammation. It has been reported by many groups that cancer patients with lymphoma who received radiotherapy were at high risk of developing mesothelioma as long as 21 years after radiation exposure (Brown *et al*., 2006; Teta *et al*., 2007). Radiation to the abdomen and thorax has also been reported to result in MM development (Amin *et al*., 2001; Travis *et al*., 2005). In a rare case of pericardial mesothelioma, the authors considered inflammation and healing, as a result from a pericardiotomy, to have a synergistic effect with asbestos in the pathogenesis of the tumor (Rizzardi *et al*., 2010).

Inflammatory prognostic and therapeutic markers: Several studies have shown that inflammatory profiles in tumors can be used as prognostic as well as therapeutic markers. Interleukin-6 (IL-6) is a multifunctional cytokine that regulates immune response and inflammation and its overproduction has been shown to underlie a number of malignancies including mesothelioma. Blocking of IL-6 signaling may be a therapeutic possibility in mesothelioma (Nishimoto, 2010). Another interleukin under investigation for prognostic and therapeutic potential in MM is interleukin-4 (IL-4). Burt *et al*. (2012) assessed tumor expression of interleukin-4 receptor α (IL-4Rα) by RT-PCR analysis in 37 Malignant Pleural Mesothelioma (MPM) specimens from patients undergoing surgical resection. Greater expression of IL-4Rα was predictive of poor survival in epithelial, but not non-epithelial subtypes of MPM patients. Additionally, the study showed that T cells within MPM tumors from eight patients undergoing Extrapleural Pneumonectomy (EPP) produced higher frequencies of IL-4 upon stimulation compared to matched T cells from blood samples. Furthermore, human MPM cells had increased STAT-6 phosphorylation and increased production of inflammatory cytokines, IL-6, IL-8 and VEGF, in response to IL-4 (Burt *et al*., 2012).

Based on research supporting asbestos-induced inflammation in the pathogenesis of MM, Kao *et al*. (2010; 2011) investigated the blood neutrophil-to-lymphocyte ratio (NLR), an index of systemic inflammation, as a prognostic factor in MM patients (Kao *et al*., 2010). The findings from the study indicated that the NLR is an independent predictor of survival for patients with MM undergoing systemic therapy. A separate study with 85 patients demonstrated that both low calretinin expression and high NLR were independently associated with poor prognosis in patients with MPM undergoing EPP (Kao *et al*., 2011). Pinato *et al*. (2012) validated the prognostic role of NLR in a large cohort of 171 MPM patients and also found the modified Glasgow Prognostic Score (mGPS), based on levels of C reactive protein and hypoalbuminemia, to be an independent predictor of survival by multivariate analysis (Pinato *et al*., 2012).

The prognostic value of tumor-infiltrating lymphocytes on survival of patients with MPM has been studied in small patient series at single institutions (Leigh and Webster 1982; Yamada *et al*., 2010). Anraku *et al*. (2008) analyzed 32 specimens from MPM patients who were treated with induction chemotherapy followed by EPP for distribution of T cells by immunohistochemical analysis. They demonstrated that the presence of high levels of CD8+ tumor-infiltrating lymphocytes was associated with improved prognosis, lower incidence of mediastinal node disease and longer progression free survival (Anraku *et al*., 2008). In another study, Yamada *et al*. (2010) investigated tumor-infiltrating lymphocytes by immunohistochemistry in MPM specimens and analyzed a subpopulation of 27 patients who underwent surgical resection. It was found that these patients showed a significantly better prognosis in correlation with a high density of CD8+ tumor-infiltrating lymphocytes (Yamada *et al*., 2010). A semi-quantitative assessment of inflammatory response on routine hematoxylin and eosin stained slides can also predict survival in patients with epithelioid MPM. A study on 175 epithelioid MPM specimens investigated inflammatory responses in tumors and stroma and suggested a positive prognostic value of inflammatory response in the stroma of epithelioid MPM. Interestingly, inflammation within the tumor, as opposed to the stroma, was associated with a vascular invasion, a poor prognostic feature (Suzuki *et al*., 2011). Collectively, these studies provide rationale for investigating immunotherapy to benefit epithelioid MPM patients.

HMGB-1, a damage associated molecular pattern protein that is involved in asbestos induced inflammation, has been demonstrated to be present in higher levels in the serum of 20 MM patients compared to 20 age-and-gender matched healthy controls (Jube and Rivera, 2012). In the same study, HMGB-1 was analyzed in 31 MM biopsies and cytoplasmic staining of HMGB-1 was shown to correlate with tumor stage, however this correlation needs to be validated due to small sample size. Additionally, inhibition of HMGB-1 in vivo by anti-HMGB-1 neutralizing antibodies

suppressed MM xenographs from specific, but not all MM cell lines evaluated. Together, the above findings support the need for further investigation of the HMGB-1 axis as a potential prognostic and therapeutic target for MM in the future.

Conventional therapy of malignant mesothelioma: Surgical resection is the only curative treatment of MPM while other treatments minimally improve response rate and overall survival. The majority of patients diagnosed with MPM are unable to undergo surgical resection because of advanced disease at time of presentation. The standard of care for these patients is chemotherapy with cisplatin plus an antifolate, such as pemetrexed or raltitrexed, which improves overall survival from 9 months to 12 months (Vogelzang *et al*., 2003; Kelly *et al*., 2011). Radiotherapy may be used for palliative care, however there is no evidence for routine use of radiation as primary therapy for MPM (Price, 2011). For patients with surgically resectable MPM, surgical options include extrapleural pneumonectomy or pleurectomy with decortication which may be combined with intracavitary chemotherapy at time of resection or systemic chemotherapy and radiotherapy (Richards *et al*., 2006; Takigawa *et al*., 2011). Unfortunately, only a small percentage of MPM patients will qualify for multimodality treatment and despite this aggressive therapy, MM recurrence is frequent.

Diffuse malignant peritoneal mesothelioma (DMPM) makes up 15-20% of MM diagnoses and like MPM, is typically diagnosed at late stage (Turner *et al*., 2012). DMPM is ultimately fatal although advances have been made in therapeutic strategies for surgically resectable disease. The combination of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy with cisplatin plus doxorubicin or mitomycin-C has been shown to improve overall survival (Baratti *et al*., 2011; Turner *et al*., 2012). Patients with inoperable DMPM may undergo systemic chemotherapy with cisplatin and pemetrexed and/or palliation surgery.

Experimental therapy of malignant mesothelioma: Inflammation presents a new avenue for the exploration of therapeutic interventions for MM. Novel therapies such as gene therapy, immunotherapy and targeted molecular therapy are under investigation through preclinical research and clinical trials (Takigawa *et al*., 2011; Vachani *et al*., 2011; Zauderer and Krug, 2012). Here we review clinical trials that target modulation of inflammatory responses in MM treatment.

MM expresses high levels of Epidermal Growth Factor Receptor (EGFR) however clinical trials have shown limited therapeutic activity of EGFR inhibitors in MM patients. A phase II clinical trial investing gefitinib, a selective inhibitor of EGFR, demonstrated this agent was not effective in the treatment of MM and overexpression of EGFR did not predict response to gefitinib (Govindan *et al*., 2005). Other tyrosine kinase inhibitors, such as imatinib and erlotinib, have been studied in phase II clinical trials and have produced negative results (Mathy *et al*., 2005; Garland *et al*., 2007). This includes a multicenter, phase II clinical trial that investigated erlotinib in combination with bevacizumab, a VEGF inhibitor, in previously treated MPM patients and showed no complete or partial responses (Jackman *et al*., 2008). Postulated resistance mechanisms to EGFR inhibitors include activation of downstream pathways of Extracellular signal Regulated Kinase (ERK) and phosphatidylinositol 3-kinase/Akt and absence of mutations in EGFR kinase domain (Garland *et al*., 2007; Velcheti *et al*., 2009). Ongoing clinical trials involving inhibition of EGFR include a multicenter, phase II study of Cetuximab, a monoclonal antibody against EGFR, in combination with standard first-line chemotherapy in MPM patients (Clinicaltrials.gov identifier: NCT0996567) and phase II study of erlotinib in malignant peritoneal mesothelioma patients who harbor EGFR kinase domain mutations (Clinicaltrials.gov identifier: NCT01592383).

Growth factors under MM clinical investigation include Insulin-Like Growth Factor (IGF) and HGF with downstream pathways involved in cell growth, anti-apoptosis, invasion and metastasis (Klominek *et al*., 1998; Zha and Lackner 2010). Cixutumumab, a monoclonal antibody to Insulin-like Growth Factor receptor 1 (IGF-IR), has been shown to delay growth of mesothelioma tumor xenographs and improve overall survival in mice (Kalra *et al*., 2012). A phase II clinical trial is evaluating IMC-A12 (cixutumumab) in pleural and peritoneal MM patients who have failed treatment with chemotherapy. The primary outcome is clinical response rate (Clinicaltrials.gov identifier: NCT01160458). Another phase II clinical trial is investigating an Anti-HGF Monoclonal antibody, (AMG) 102, in combination with pemetrexed disodium and cisplatin in MPM patients with a primary outcome of progression free survival (Clinicaltrials.gov identifier: NCT01105390).

Tumor-homing peptide asparagine-glycine-arginine (NGR)-human tumor necrosis factor alpha (NGR-hTNF) is being investigated for its role to induce antitumor activity in MM patients. This agent is a modification of TNF-α to reduce systemic toxicity and selectively bind aminopeptidase N isoform which is overexpressed on tumor blood vessels (Gregorc *et al*., 2010). A multicenter, nonrandomized, single agent, phase II clinical trial in previously treated MM patients

showed NGR-hTNF was tolerated and resulted in disease control in about half the patients (Gregorc *et al*., 2010). In a randomized, double blinded, phase III clinical trial of NGR-hTNF plus Best Investigator's Choice (BIC) versus placebo plus BIC was initiated in MPM patients who have progression of disease after treatment with pemetrexed-based chemotherapy. Overall survival is the primary outcome measure and the estimated completion of the study is February of 2013 (Clinicaltrials.gov identifier: NCT01098266). NGR-hTNF is also being evaluated as maintenance therapy in MM. A randomized, double blinded, phase II clinical trial is investigating the efficacy of NGR-hTNF plus best supportive care versus placebo plus best supportive care as maintenance therapy in advanced MPM patients with non-progressing disease immediately following six cycles of pemetrexed-based chemotherapy. The primary end point is progression free survival and the total enrollment is expected to be 100 patients (Clinicaltrials.gov identifier: NCT01358084).

Interferons are immunostimulatory cytokines that enhance anti-tumor responses. Clinical trials involving gene therapy in MM patients that aimed to deliver interferon beta (IFN-β) through adenoviral vectors by intrapleural administration have shown to be feasible and resulted in induction of humoral immune responses and positive clinical responses. However, the development of neutralizing antibodies remains a limitation for this therapeutic method (Sterman *et al*., 2007; 2010). Other ongoing clinical trials involving interferon therapy and MM include a phase I clinical trial investigating the gene transfer of IFN-α (Clinicaltrials.gov identifier: NCT01212367) and a phase 0 clinical trial evaluating intrapleural adenovirus mediated IFN-α in combination with first-line or second-line chemotherapy (Clinicaltrials.gov identifier: NCT01119664).

TGF-β is a multifunctional cytokine that is overexpressed in MM and may function as a tumor promoter through immunosuppression. TGF-β has received attention as a therapeutic target based on in vivo studies that demonstrated blocking of TGF-β by neutralizing antibodies, TGF inhibitors and other methods resulted in MM tumor inhibition (Marzo *et al*., 1997; Suzuki *et al*., 2004; 2007). A phase II clinical trial investigating the use of an anti-TGF monoclonal antibody, GC1008, in relapsed MPM patients is in progress. The primary outcome is assessment of survival rate progression at 3 months (Clinicaltrials.gov identifier: NCT01112293). Activin like receptor kinase 1 (ALK-1) is a member of the TGF-β type I receptor family and is involved in angiogenesis (Oh *et al*., 2000). An ongoing phase II clinical trial is investigating an anti-ALK-1 monoclonal antibody, PF-03446962, in advanced stage MPM patients. Response rate is the primary outcome and estimated completion of the study is June 2014 (Clinicaltrials.gov identifier: NCT01486368).

IL-2 is a proinflammatory cytokine and promotes development of cytotoxic T lymphocytes. Past clinical trials have shown that intrapleural administration of IL-2, as opposed to subcutaneous and intravenous route, has the greatest potential for anti-tumor activity with acceptable toxicity when administered alone or in combination with multimodality therapy (Astoul *et al*., 1998; Castagneto *et al*., 2001; Mulatero *et al*., 2001; Lucchi *et al*., 2007). Ali *et al*. (2009) conducted a clinical trial that demonstrated intrapleural preoperative IL-2 treatment in 60 MPM patients resulted in increased tryptase mast cells and tumor infiltrating lymphocytes compared to 30 untreated MPM patients undergoing surgical resection. Additionally, the study proposed that the above immunological parameters may be used as predictors of outcome in MPM patients treated with IL-2 (Ali *et al*., 2009).

Mesothelin has become the target for antibodies against MM as this cytoplasmic membrane glycoprotein is highly expressed in epithelioid subtype MM tumors with limited expression on normal human tissues (Kelly *et al*., 2011). Currently, there are multiple anti-mesothelin antibodies under investigation in clinical trials for the treatment of MM. MORAb-009 is a high affinity monoclonal antibody for mesothelin that is implicated to disrupt the cell adhesion function of mesothelin and induce cell mediated cytotoxicity (Hassan *et al*., 2007). A phase I clinical trial was conducted in patients with high mesothelin expressing advance solid tumors which included 13 MM patients out of 24 patients enrolled. The study demonstrated MORAb-009 was safe and well tolerated. Of 20 patients who completed at least one cycle of MORAb-009, 11 had stable disease although no objective clinical responses were observed (Hassan *et al*., 2010). An open label, phase II clinical trial is now in place which investigates MORAb-009 in combination with cisplatin and pemetrexed in patients diagnosed with epithelioid or biphasic mesothelioma subtypes without prior systemic chemotherapy or radiotherapy. The primary outcome is progression free survival (Clinicaltrials.gov identifier: NCT 00738582). SS1P is a recombinant immunotoxin made up of an anti-mesothelin Fv fragment linked to a truncated Pseudomonas exotoxin (Chowdhury and Viner, 1998). A single center, phase I clinical trial evaluating dose escalation of SS1P with concurrent administration of cisplatin and pemetrexed in unresectable epithelioid MPM patients is underway (Clinicaltrials.gov

identifier: NCT01445392). Additionally, SS1P is being investigated in a phase I clinical trial in mesothelioma patients with immune suppression drugs, pentostatin and cyclophosphamide, as an attempt to decrease the immunogenicity of SS1P (Clinicaltrials.gov identifier: NCT01362790).

CONCLUSION

MM is a difficult to treat cancer associated with asbestos exposure and requires a multidisciplinary approach such as the combination of surgery, chemotherapy and radiation. It is not realistic to expect that any single intervention will be enough to treat this aggressive cancer. Inflammation plays an important role in MM development and improving our understanding of this phenomenon will help identify inflammatory biomarkers that are useful predictive or prognostic tools as well as facilitate the development of novel treatments for this deadly disease.

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