Review

Mouse models of malignant pleural effusion

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SUMMARY. **Malignant pleural effusion (MPE) is a common complication of advanced malignancies, particularly lung and breast cancer. The survival time of patients with MPE is often short, with poor quality of life. The pleural space normally contains a thin film of fluid that is regulated by the balance between production from systemic blood vessel filtration and lymphatic absorption. Tumour-induced disturbances of the pleural fluid production and clearance processes result in the development of MPE. Until recently the specific mechanisms underlying pleural fluid accumulation were poorly defined because studies of MPE pathogenesis were limited by a lack of animal models that could reproduce the pathobiology of human MPE. During the past decade, various research groups have established experimental models that mimic human pleural malignancies, including mice models that require either immunocompromised or immunocompetent mice for propagation of human or murine cancer induced-MPE, respectively. The experimental modelling of MPE has provided new insight into the biological behaviour of tumour cells and tumour-host interactions in the pleural cavity, paving the way for improved management of this end-stage condition.** ***Pneumon 2013, 26(3):216-222.***

INTRODUCTION

Malignant pleural effusion (MPE) is a significant clinical problem that affects 7,000 patients annually in Greece alone. In developed countries, MPE is the second most frequent cause of exudative pleural effusion after parapneumonic effusion[1,2]. Its incidence is high (500-700 cases per 100,000 residents per year) almost reaching lung cancer in incidence[3].

MPE is caused by primary or metastatic tumours that enter the pleural cavity, but is mainly a metastatic disease[4]. Lung cancer is the most common metastatic tumour to the pleura, accounting for approximately 37.5 % of all MPE[2]. Breast cancer accounts for 17% and blood malignancies, such as lymphoma, for 11.5%. Neoplasms of the gastrointestinal and genitourinary tractsfollow, wiht rates reaching 7% and 9.5% respectively, but effusions from an unknown primary site account for 11% of all MPE[5].

MPE signifies advanced systemic disease and is associated with a low

survival rate and poor quality of life. Patients at diagnosis of MPE can expect a median survival of about 3-6 months and are likely to suffer from dyspnoea, cough and chest pain, usually accompanied by weight loss, anorexia and severe fatigue.

Currently, the primary goal in MPE management is effective palliation of the associated respiratory symptoms. This is achieved by drainage of the pleural fluid and/or prevention of its reaccumulation. Treatment options include thoracentesis, pleurodesis, pleuroperitoneal shunting, pleurectomy and tunnelled pleural catheters[6].

PATHOGENESIS OF MPE

The pleural membrane is thin and moist and it covers the external surface of the lungs and the internal surface of the chest wall. It consists of two layers, visceral and parietal, which enclose the pleural cavity. The pleural cavity normally contains a small amount of fluid which is called pleural fluid[7].

The pleural fluid is produced by diffusion from the capillaries of the parietal pleura and is removed by lymphatic drainage through the stomata of the parietal pleura[8]. Under normal conditions, the body has the ability to remove up to 30 times the usual rate of fluid formation.

A classical cause of MPE formation is reduced pleural fluid drainage due to tumour-associated blockage of the local lymphatic outflow[9,10]. Recent findings, however, show that the basic prerequisite for MPE formation is interaction between pleural-based tumour cells and the vasculature and immune system of the host that results in increased net fluid production through enhanced plasma extravasation into the pleural space[11].

ANIMAL MODELS OF MPE

Development of a suitable animal model

Over the past decade, significant progress has been made in research on MPE, but the relevant findings have yet to be translated into advances in treatment. Selection of a simple and feasible tumour-bearing animal model provides one means of studying the pathogenesis of and potential forms of treatment for MPE. Over the years, various animal models of MPE have been developed that have provided insight into the mechanisms of effusion formation.

Most animal studies on MPE have been performed using laboratory mice, which present several advantages including 90% genome homology with humans, small body size, rapid tumour growth, with the availability of many inbred and genetically engineered strains and an entirely sequenced genome. Occasionally, larger animals such as rabbits and rats have been used, which provide easier experimental manipulation and more adequate amounts of biological material for examination.

Effective cancer cell or tissue inoculation into the pleural cavity can be achieved using various different implantation methods:

Thoracotomy, where orhtotopic implantation of freshly isolated malignant tissues is effected. The tumour tissue is tied into the visceral and parietal pleura. This method offers a high uptake rate of the cancer; but it is an invasive and painful method that may have negative effects on the respiratory system of the animal[12,13].

Intravenous injection, with blood-borne translocation of tumour to the lung vasculature and lung/pleural outgrowth. Intravenous injection of human lung adenocarcinoma cells produces multiple lung lesions, pleural metastasis and effusions[14-16]. This approach resembles human cancer metastasis to the pleura, but it carries a high mortality rate, and interpretation of MPE is achieved only by using the specific lung adenocarcinoma cell lines.

Intrapleural injection, where a small volume of cultured cancer cell lines is delivered into the pleural cavity via a small skin incision in the left posterolateral thoracic area. This injection method results in local implantation of tumour in the chest wall, mediastinum, lungs and diaphragm. Pleural effusions (usually bloody exudates) develop a few weeks after inoculation, mimicking the advanced stage of human disease[17]. This method is not associated with mortality as it requires minimal surgery and has the advantages of on-site confirmation of orthotopic tumour cell delivery to the pleura and is of reliable reproducibility. For these reasons, intrapleural orthotopic implantation models prevail for the study of MPE.

The immune status of the animal used may strongly influence tumour growth and progression *in vivo*. Immunodeficient animals have been extensively used for modelling MPE. Various types of xenogenic tumour cells have been successfully introduced into the pleural space of immunodeficient mice or rats, producing subsequent effusion formation[14,15,18-20]. These animal models, however, do not represent the real tumour in a real environment, since MPE development is the result of a complex interaction between the tumour and the host immune response and it is important that the two interacting biological subjects use the same 'species-specific language' to ensure

that the biological phenomenon emulated by the model is fully developed.

MPE models using immunocompetent hosts implanted with syngeneic tumours can better simulate human MPE[17,21,22]. Specifically, the intrapleural injection of syngeneic cells in immune-intact mice is highly relevant to human disease and all the treated mice develop MPE[17]. This model has the advantage that the host immune system is intact, so that the tumour microenvironment will mirror as closely as possible the human situation, and the role of specific molecules and genes in tumour development and progression can be explored at all stages. A disadvantage is that mice tumours are used and may behave differently from the human tumour with regard to the therapeutic response.

Individual animal models of MPE

At present there is only one animal model of spontaneous MPE generation[28]. This model rests on a combined conditional knockout mouse with pleural mesothelial cells specifically deficient in genes frequently found altered in human mesothelioma (Nf2; Ink4a/Arf; p53). The resulting mouse provides a conditional model for malignant mesothelioma with pleural effusion formation.

The majority of animal models of MPE have been developed in immune-deficient hosts. The immune-deficient animals used include severe combined immunodeficient (SCID)[18,19] and athymic (nude; natural cytotoxicity receptor, NCR-deficient)[14,15] mice, and immune-deficient rats[20]. Yano and colleagues (2000) established a model using athymic nude mice to which human lung adenocarcinoma cells were delivered intravenously, causing lesions in the lung parenchyma and invading the pleura and producing MPE[14]. This model was replicated successfully by another research group[15]. The intravenous delivery of tumour cells mimics hematogenous metastasis but as in the models a defined primary tumour as a source of metastatic spread is missing implantation of the tumour in the organ specific orthotopic site could be more relevant to the clinical situation.

Boehle and colleagues established a model of orthotopic xenotransplantation of human lung cancer with subsequent MPE formation. Specifically, they injected intrapulmonary and intrapleurally into SCID mice human adeonocarcinoma, squamous cell carcinoma and undifferentiated large cell carcinoma cells with an engraftment rate of 80% to 100% (Boehle 31). Similar models of orthotopic implantation in SCID mice have been used for inducing MPE by other research groups[19,25,29]. Human mesothelioma cell lines or human lung adenocarcinoma cell lines were implanted intrapleurally in SCID mice, following which tumour growth and development of malignant pleural effusion were observed. In addition, one orthotopic model system has also been developed in immunodeficient rats, which developed MPE after inoculation of human lung adenocarcinoma cells directly into the thoracic cavity[20].

Both athymic nude and SCID mice with human tumour cell implantation have contributed significantly to the understanding of basic aspects of MPE. Recent information regarding the important influence of the tumour microenvironment on tumour progression and growth, however, has led to greater reliance on immunocompetent models. Kimura and colleagues implanted syngeneic meth A fibrosarcoma into the pleural space of Balb/c mice[21]. An MPE model has also been established in immunocompetent New Zealand white rabbits by intrapleural inoculation of rabbit VX2 sarcoma[22,30,31]. Another highly relevant and 100% reproducible animal model which was developed by Stathopoulos and colleagues involves the intrapleural injection of Lewis lung carcinoma (LLC) cells to syngeneic wild type C57BL/6 mice[17]. The implantation and growth of tumours in the pleural cavity triggers a host immune response, evident by a mixed inflammatory cell component in the pleural fluid that is similar to the inflammatory cells found in human MPE. This model has been replicated and further developed by the same and other research groups[29,32-34]. With the aim of establishing mouse models of MPE induced by additional histological types of cancer, our group has in recent years developed syngeneic mouse models for MPE development using colon adenocarcinoma and mesothelioma[29,32]. The features of the above MPE models are summarized in the following table.

Stages of MPE formation

Intrapleural injection of malignant cells in immunocompetent mice leads gradually to the formation of MPE in approximately 14 days[17,29,32,36,37]. This phenomenon can be divided into four phases:

Phase 1. Takes place from day 0 to day 4. During this phase tumour implantation occurs. Day 0 is the day of the intrapleural injection of tumour cells into the mice. Thereafter tumour cells start proliferating and forming small tumour foci on the surface of the visceral and parietal pleura.

Phase 2. Takes place from day 5 to day 8 and represents an inflammatory phase of MPE development. Tumours are growing and secrete cytokines and chemokines that

AUTHOR	YEAR	SPECIES	TUMOR CELL/ TISSUE	INTRODUCTION ROUTE	IMMUNE COMPETENCE	ADVANTAGES	DISADVANTAGES
Yano *et al*	2000	mice	Human lung adenocarcinoma cells	intravenously	Athymic nude	Mimics hematogenous metastasis	A defined primary tumour as a source of metastatic spread is missing
Boehle *et al*	2000	mice	Human adenocarcinoma squamous cell carcinoma and undifferentiated large cell carcinoma cells	Intrapulmonary and intrapleurally	SCID	Injection of human cancer cell line	Not taking into account the important influence of the tumour microenvironment on tumour progression and growth
Kimura *et al*	2000	Balb/C mice	Syngeneic methA fibrosarcoma	intrapleurally	Immunocompetent	Orthotopic implantation	Mouse cancer cell line
Ohta *et al*	2001	rat	Human PC14 lung adenocarcinoma	Intrapleurally and subpleurally	immunodeficient	Injection of human cancer cell line	Not taking into account the important influence of the tumour microenvironment on tumour progression and growth
Hatton *et al*	2002	New Zealand white rabbit	Rabbit VX2 tumour	Thoracic surgery	immunocompetent	Combined transplantation of tumour cells and stroma	Thoracic surgery is time-consuming and with high mortality
Yeh *et al*	2006	mice	Human PC14PE6/ AS2 lung adenocarcinoma cells	Intravenously and intraperitoneally	Nude	Injection of human cancer cell line	The behaviour of cancer cells and the peritoneal clearance fluid may be different from the pleural
Edakuni *et al*	2006	mice	Human mesothelioma cell line	Subcutaneously and intrapleurally	SCID	Injection of human cell line	Not taking into account the important influence of the tumour microenvironment on tumour progression and growth
Jongsma *et al*	2008	FVB mice	Genetic model	Spontaneous development of mesothelioma	immunocompetent	Spontaneous MPE development	Requires time to develop MPE
Stathopoulos *et al*	2006	C57BL/6 mice	Lewis lung carcinoma (LLC) cells	intrapleurally	immunocompetent	Takes into account the important influence of tumour -host interactions on MPE development	Mouse cancer cell line

attract a mixed inflammatory cell population. Pleural exudates start forming and at the end of this phase the pleural fluid volume is low, but contains high concentrations of tumour and host derived inflammatory mediators and immune cells.

Phase 3. Takes place from days 9-12. During this phase increased vascular permeability ensues. The pleural tumour foci secrete angiogenic cytokines that render vessels hyperpermeable. Plasma fluid and proteins leak into the pleural cavity. Pleural fluid accumulates and starts appearing haemorrhagic, as occurs in humans at an advanced stage of the disease.

Phase 4. Takes place from day 13 until death. In this phase the enriched pleural fluid feeds tumour foci leading to accelerated tumour growth and spread to neighbouring structures such as lung, chest wall and diaphragm. Serious respiratory distress occurs in the mice as the lungs are compressed by the abundant tumour and fluid formation, leading to severe dyspnoea and death.

Assessment of MPE formation

The general status of the experimental animal reflects the tumour progression. As MPE develops the mice gradually lose weight and present impaired physical activity. At advanced stages of MPE mice appear to have dyspnoea and become cachectia. Th, daily observation of experimental mice is necessary, along with measuremenn of the body weight at regular intervals. Pleural fluid, tumours and neighbouring tissues are collected at necropsydfor processing anr analysis using a variets of techniques.

The primary end point of experimental MPE is the pleural fluid volume, appearance ans cellular content. The volume of pleural effusion is measured using calibrated syringes or pipettes. Murine MPEs are exudativ, and blood,; they do not coagulate and they reace a volume of between 200 μl and 1,500 μl. Another important end point is the determination of the pleural tumour numbers and size. Visceral and parietal pleural implantations can be enumerated under a dissecting microscope. A more elegant method for pleural tumour burden evaluation is stereology[23,35]. Several non invasive imaging techniques, including(computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), *in vivo* bioluminescence)and others, have been used extensively to monitor tumour progression and pleural fluid formation in the living animals[24-26].

Additional end points that are determined are tumour induced inflammation, angiogenesis and vascular hyperpermeability in the pleural cavity of the experimental mice. Most of the nucleated cells in the pleural fluid,rapart from the tumour and mesothelial cells, are monocytes, neutrophils and lymphocytes. Thelinflammatory cellssand the inflammatory and angiogenic mediators are measured in the pleural fluid, tumour mass and peripheral blood of animals with MPE. In addition, increased angiogenesis in a tumour leads to its accelerated growt, and pleural tumour tissue can be examined for proliferation and apoptosis rates. Plasma leakage from pleural vessels in experimental MPE is quantified using various different methods, such as Evan's blue dye pulse and chase, modified Miles assay or comparison of the total protein contentnin pleural fluid and serum[17,27].

TRANSLATIONAL ADVANCES IN MPE

MPE is a lethal disease that necessitates devoted translational research effors in order for improvements to be introduced into the clinical setting. In the past decade, several preclinical studies that employed the experimental models of MPE above have yielded significant insighs into the pathobiology of the condition.

Although most of the earlier work on MPEhwas performed in immunodeficient mice, the development of immunocompetent models represents a significant step forward for translational pleural research, as host tumour interactionseplay a key rols in MPE formation and progression. Using these models the roles of significant mediators and biological pathways in effusion formation havs been uncovered. The most prominent mediators and pathways involved in this process appear to be VEGF, IL-6/Stat3, MCP-1, Spp1, angiopoietin/Tie2 and TNF/NF-κB signalling[14,15,17,33,36-38]. These tumour-originated gene products function to promote tumour growth in an autocrine manner and also to co-opt host cell populations intimately linked with pleural tumour progression and fluid accumulation, presenting new therapeutic targets in preclinical models and in human pleural effusions.

In addition, some studies have examined the efficacy of novel therapies in both immunocompetent and immunodeficient mouse models, and have provided insights into potential future forms of treatment fot MPE. In this contexd, the synergism of interleukin IL-12 and IL-15 blockade was shown to be beneficial against experimental MPE[21], inhibitors of topoisomerase II were applied[39] and VEGF-receptor tyrosine kinase inhibition was performed[14] at the preclinical level. Bortezomib (an indirect inhibitor of NF-κb activation) and zoledronic acid (an aminobiphosphonate that exerts potent antitumour

effects) were also found to exert beneficial effects against mouse MPE by inhibiting tumour-specific NF-κβ and Ras signalling, respectively [40,41]. Recent studies using a sulindac derivative showed promising effects on intrapleural tumour dissemination via down-regulation of pleural vascular permeability [42]. CCL2 and CCL12 neutralization alone or in combination were observed to exert an inhibitivy impact on thE development of MPE induced by murine and human adenocarcinomas [29].

CONCLUSION

MPE is a common and distressing condition associated with advanced-stage cancer. The development of various animal models of MPE has paved the way for understanding the genetic and molecular pathogenesis of this disease.eFurther research efforts are now needed to establish effective and targeted therapeutic strategies based on the findings in the animal models.

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