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Malignant human cell transformation of Marcellus Shale gas drilling flow back water☆

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ABSTRACT

The rapid development of high-volume horizontal hydraulic fracturing for mining natural gas from shale has posed potential impacts on human health and biodiversity. The produced flow back waters after hydraulic stimulation are known to carry high levels of saline and total dissolved solids. To understand the toxicity and potential carcinogenic effects of these wastewaters, flow back waters from five Marcellus hydraulic fracturing oil and gas wells were analyzed. The physicochemical nature of these samples was analyzed by inductively coupled plasma mass spectrometry and scanning electron microscopy/energy dispersive X-ray spectroscopy. A cytotoxicity study using colony formation as the endpoint was carried out to define the LC_{50} values of test samples using human bronchial epithelial cells (BEAS-2B). The BEAS-2B cell transformation assay was employed to assess the carcinogenic potential of the samples. Barium and strontium were among the most abundant metals in these samples and the same metals were found to be elevated in BEAS-2B cells after long-term treatment. BEAS-2B cells treated for 6 weeks with flow back waters produced colony formation in soft agar that was concentration dependent. In addition, flow back water-transformed BEAS-2B cells show better migration capability when compared to control cells. This study provides information needed to assess the potential health impact of post-hydraulic fracturing flow back waters from Marcellus Shale natural gas mining.

Introduction

Natural gas is believed to possibly be a bridge to transitioning from coal dependence. Currently natural gas fuels nearly 40% of the U.S. electricity generation, and the Marcellus Shale formation in the Appalachian Basin is on the forefront of gas-shale drilling for natural gas production in the United States (Pritz, 2010). Mining natural gas is not new, but the volume has soared in recent years because the new technique of high-volume horizontal hydraulic fracturing (HVHHF). The concern surrounding the environmental, public health and social impacts of this method has increased accordingly. HVHHF is an advanced technology that injects water, sand, and other ingredients at very high pressure vertically into a well about 6000 to 10,000 ft deep (Penningroth et al., 2013). The high pressure creates small fractures in the rock that extend out as far as 1000 ft away from the well. The pressure is reduced after the fractures are created, which allows water from the well to return to the surface, also known as flow back water (Veil, 2010). The flow back water contains complex proprietary chemical mixtures, but also naturally occurring toxins such as metals, volatile organics, and radioactive compounds that are destabilized during gas extraction (Warner et al., 2012). On average, about 5.5 million gallons of water is used on average to hydraulically fracture each shale gas well, and 30% to 70% of the volume returns as flow back water (Veil, 2010). Currently discharge options of flow back water are: inject underground through an onsite or offsite disposal well; discharge to a nearby surface water body; transport to a municipal wastewater treatment plant or publicly owned treatment works; transport to a commercial industrial wastewater treatment facility; and/or reuse for a future hydraulic fracturing job either with or without some remediation (Pritz, 2010). Some commercial wastewater disposal facilities accept flow back and discharge the water after treatment under their own national pollutant discharge elimination system permits (Veil, 2010).

Metal pollution is a serious problem as they are taken up readily in the digestive tract and exhibit harmful effects on many tissues (Alomary et al., 2013; Rasmussen et al., 2013). Barium and strontium

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are abundant in the Marcellus Shale formation, and are easily dissolved and transported in wastewater after gas drilling activity (Pritz, 2010), which could potentially pose a threat to drinking water (EPA).

In contrast to the increased support of drilling and exploration by U.S. government agencies and rising concerns of impact on human and animal health within close proximity of the drilling sites (Bamberger and Oswald, 2012), knowledge of the health risks associated with the gas drilling wastewater is sparse. The question posed here is whether flow back water specific to the Marcellus Shale malignantly transforms cells, and if it does, what's the mechanism underlying tumorigenic potential of produced flow back water.

It has been challenging yet critical to choose a proper human cellular model to address this question. Immortalized human bronchial epithelial cells (BEAS-2B) have been widely used as a malignant cell transformation model to estimate the carcinogenesis capability of various environmental toxicants (Liao et al., 2007; Chang et al., 2010; Son et al., 2012; Yang et al., 2013). It has wild-type and functional p53 gene expression due to the loss of SV40 in passages after immortalization process, which provides a low spontaneous anchorage free growth, a quality of good cellular model for malignant cell transformation analysis (Lehman et al., 1993).

In this study we employed BEAS-2B as well established models (Lee et al., 1993; Chen et al., 2006; Sun et al., 2011; Passantino et al., 2013) to investigate the malignant cell transformation of Marcellus Shale gas drilling flow back water.

Material and methods

Cell culture and exposure

BEAS-2B (ATCC, Manassas, VA) cells were cultured as previously described (Sun et al., 2011; Passantino et al., 2013) at 37 °C in a humid 5% CO_2 atmosphere. BEAS-2B cells were seeded at 3×10^5 into 25 cm^2 polystyrene tissue culture flasks. The cells were treated with filtered (0.22 μm filter) produced flow back water collected from Bradford County, PA (a generous gift from Dr. Carl Kirby and Dr. Judy Zelikoff), and diluted with the appropriate medium to 0.13%, 0.25%, 0.5%, 1%, 2%, 4% or 8% (v/v). Control cells received distilled water or filtered water from a pristine lake located in Sterling Forest (SF), NY, diluted with the designated medium to 4% (v/v). The cells were cultured for various time intervals as indicated. Every 3 to 4 days, the cells were trypsinized, counted, and re-seeded into fresh 25 cm^2 flasks at a density of 3×10^5 viable cells per flask, and provided fresh media with the appropriate concentration of the flow back water sample.

Scanning electron microscopy (SEM)/energy dispersive X-ray spectroscopy (EDX)

The water samples were briefly sonicated in bath sonication for 1 min to make sure the solution was uniform. Both high density and low density samples were prepared. High density water samples were prepared with one drop of 100 μL of each sample dried on carbon tape in a class 100 clean room. For low density samples, 100 μL of each sample was spin coated onto a carbon tape surface pre-mounted to a SEM sample holder at 200 rpm before being air dried overnight in a class 100 clean room. Field Emission Scanning Electron Microscopy (FEI, The Netherlands) and Energy Dispersive Spectrometry (model Genesis 60S, by EDAX Company, USA) were performed to identify any particles in the sample and the chemical components of those particulates were analyzed by EDX.

Inductively coupled plasma mass spectrometry (ICP-MS)

A volume of 0.1 mL of each sample including flow back water and their filtrates was ionized in tubes with 1 mL HNO_3 (70%) at 140 °C for 5 h. Concentrations of the heavy metals were determined by an ICP-MS (Perkin Elmer, Warsaw, Poland). BEAS-2B cells after 5 weeks of treatment with flow back water at 0.5% (v/v) were trypsinized and counted to determine the total cell number. The cell pellets with same amount of cells were then mixed with 3 mL of HNO_3 (70%) and incubated at 80 °C for 48 h, followed by cooling for 1 h to room temperature. After cooling, 3 mL of hydrogen peroxide (30%) was added to each tube, followed by incubation of the solution at 80 °C for 3 h. After suitable dilution of the digested materials with ultrapure water, levels of elements in the samples were determined by ICP-MS.

Colony formation and soft agar assay

Following treatment, BEAS-2B cells were trypsinized and counted using a hemocytometer to determine viability. Colony formation and soft agar assay were then conducted, and cells that exhibited anchorage free growth were collected for wound healing assay according to previously published procedure (Passantino et al., 2013); for detailed information, please see supplemental materials.

Cell migration assays

Matrigel (BD Biosciences, Bedford, MA) was reconstituted on the top surfaces of Transwell membranes at 100 μg protein/cm^2 of surface area. Transformed BEAS-2B cells (5×10^4 in 100 μL) were added to the upper chamber in serum free medium supplemented with 0.5 μM plasminogen. The bottom chamber contained DMEM supplemented with 10% FBS. The cells were allowed to invade for 24 h at 37 °C, at which time the Matrigel and cells that were associated with the top surfaces of the membranes were removed with cotton swabs. Cells that penetrated through the Matrigel to the underside surfaces of the membranes were fixed and stained with 0.1% Crystal Violet. Cells on the lower surface of the filter were enumerated using an ocular micrometer. Five fields were counted. Each experiment was performed twice with triplicate samples.

Wound healing assay

Cells (2×10^5) from each clone extracted from soft agar were plated into 35 mm culture dishes with a grid etched into the bottom. The cells were cultured in 1× DMEM complete media until 100% confluent (4 days). The media were then replaced with 1× PBS, and a single scratch was made across the monolayer using a 1 mL pipette tip held perpendicular to the plate bottom. The plate was washed twice

Table 1
ICP-MS analysis of filtered Marcellus Shale flow back.

Concentration (mg/L)	Well 1	Well 2	Well 3	Well 4	Well 5	SF
Al	0.301	0.044	1.268	1.102	1.057	N.D.
B	0.315	0.252	0.408	0.388	0.370	N.D.
Ba	452.4	474.6	596.5	606.8	701.6	N.D.
Bi	0.110	0.164	0.180	0.158	0.059	N.D.
Ca	470.7	974.9	707.8	608.9	502	1537
Co	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Fe	3.506	1.343	4.491	N.D.	N.D.	N.D.
K	23.43	237.4	180.8	203.4	345.1	0.248
Mg	52.89	91.70	109.1	115.1	105.0	41.20
Mn	0.341	0.357	2.58	0.43	0.34	N.D.
Na	61.48	60	62.2	57.94	90	91.62
S	8.74	70.69	9.884	9.18	14.5	0.781
Si	0.675	0.931	1.762	0.713	0.759	N.D.
Sr	183.3	281.2	361.4	353.4	339.7	1.01
Ti	0.079	0.067	0.045	0.067	0.074	0.008
V	0.206	0.267	0.441	0.512	0.457	0.006
W	1.926	3.419	9.343	N.D.	N.D.	3.28
Zn	0.062	0.049	0.054	0.003	0.030	59.02
Zr	0.066	0.073	0.033	0.008	N.D.	N.D.

N.D.: not detected.
SF: pristine lake water from Sterling Forest.